

Welfare cannot be made to go away

Most governments have taken fright at the growing cost of welfare and, perhaps inevitably during a recession, are canvassing mean devices for containing costs. But welfare is inescapable in a modern state.

FORTY per cent of the British government's budget goes on welfare in various forms, from free medical care through the National Health Service to pensions for the elderly and unemployment pay for those who have lost their jobs. It is much the same in the United States, where what are called 'entitlements' mount inexorably from year to year and where the new president's wife, Mrs Hillary Rodham Clinton, is saddled with responsibility for making health care accessible to all. The same is true of France and Germany (where the cost of medical care, mostly covered by 'insurance', is 8.7 per cent of gross domestic product). Then in Scandinavia in the past few years, governments have been chipping away at what was once the most generous welfare system in the world: tax rates became uncompetitively high. What paradox ensures that the cost of welfare comes to seem insupportable in the best-off communities? And what will persuade them that the seeming paradox is not a paradox at all?

The first principle is one that has been amply demonstrated in the past two centuries by the experience of the now-industrialized countries of the world (Japan excepted): civility, and in particular seemingly relations between rich and relatively poor, require that the very poor should have food and shelter and should not die when medicine might save them. Moreover, given that even the best-intentioned charities are necessarily idiosyncratic, it is proper that the source of welfare should be government, usually central but sometimes regional or even local. That rich communities find the burden of welfare heaviest is then no surprise, for the cost of relieving poverty is not an absolute sum, but one that necessarily increases with general prosperity. To know that, it is sufficient to observe that in many poor countries, too poor to afford welfare programmes, the majority of the population might be held eligible in Europe or North America.

The second principle is that, although welfare is rightly financed and administered by governments, no particular scheme of benefits (in Britain) or entitlements (in the United States) should be regarded as a right. The essential is merely that all eligible people should be dealt with on an equal footing. But what is then to be made of the pensions paid to elderly people, who may claim that the payments they are compelled to contribute during their working lives should be compared to insurance premiums that earn them a pension when they cease to work? That belief attaches notably to the Social Security system in the United States, partly because

of the way in which contributions are notionally added to an identifiable fund whose adequacy is regularly assessed by actuaries. Mystifyingly, social security pensions are also free from tax, which fosters the illusion that they are something special. But the belief is a myth. Pensions are, or should be, what a sympathetic government calculates to be necessary to keep the poorest old people in seemingly comfort; they should be paid to everybody, but should be taxable as a matter of course.

Governments should not be surprised that the cost of medical care is increasingly alarming. Having willed the technology of longevity (by the research they sponsor), governments can hardly complain that the fruits of their success are too expensive. What the National Health Service has done for the civility of British life over nearly half a century is to show that the very young and the very old need not be disadvantaged by circumstances, and that the chronically sick can similarly be helped to a seemingly way of life. The social benefits are incalculable (but somebody should nevertheless try to calculate them). The British system also shows that ordinarily healthy and particularly middle-class people are best able to win what they need from the health services. (By comparison, the psychiatrically disabled are awkward customers.) Mrs Clinton's best solution would thus be an extension of the Medicare and Medicaid schemes to cover the 30 million or so uninsured people in the United States — coupled with the cancellation of tax exemption for health insurance premiums.

But does not welfare engender dependence, undermining people's will to fend for themselves? The hypothesis may be correct, but the answer is not to abandon welfare nor to impede access to it. The potentially more constructive solution of giving people skills that will help them to find jobs has been found, in British experience, to be difficult and expensive to arrange — and, in the middle of a recession, ineffective. The idea that welfare recipients might be required to work at social tasks that otherwise would not be done is more appealing, but even more difficult to arrange. Despite schemes called 'workfare' in the United States (in Arkansas, among other states), enthusiasm for this solution should be tempered by the knowledge that a non-job can be as dispiriting as no job. So, while governments have some room in which to manoeuvre (by acting as monopoly purchasers of some services, for example), most had better learn to live with the notion that welfare has come to stay. □



University competition

Britain's newly diverse universities will be more vigorous rivals for each other's funds in years to come.

Nor much is to be gleaned from the distribution of funds for the support of research at English universities announced last week (see page 4), except that the Matthew Principle ("to him who hath shall be given") continues to apply. One difficulty is that funds are now separately distributed to institutions of higher education in Scotland, Wales and Northern Ireland. Another is that the institutions previously called polytechnics are now mostly called universities. A third is that the English funding council has begun identifying separately its financial support for teaching and for research. Inevitably, the universities that have done well in their research allocations will be looking for even greater largesse to come. But may they be disappointed?

The new allocations derive from the exercise, occupying the past two years, in which the volume and quality of research at the traditional universities has been assessed by the few objective measures that exist. Mercifully, it has nevertheless been agreed that the full rigours of the Matthew Principle should not apply, and that even universities with little research should enjoy some research support. Otherwise, the research universities would be forever isolated from the competition less distinguished institutions can and will provide.

The competition may come more quickly than the research universities expect. There is a hint of that in the figures announced last week; some of the old polytechnics or 'new' universities will be more generously provided with research support than they were before. They will complain, with some justice, that, even now, their teachers will have to spend more time teaching than would be thought acceptable at the traditional universities, but at least they will now be more able than in the past to recruit people likely to win favours and funds. It would also be dangerously complacent of the research universities to suppose that the old polytechnics are no intellectual threat.

There is a second enemy of complacency in the traditional universities — the still uncertain outcome of the government's labouring on a new policy for harnessing research in Britain to 'wealth creation'. Although there are the strongest reasons why support for basic research should continue on something like its present scale, it seems unlikely that the mechanisms of research support will be left unchanged. Moreover, the greater diversity of institutions of higher education with which Britain is now endowed should be an asset, at least if they are able to compete for students, teachers and funds on equal terms.

The emergence of the new universities has inevitably occasioned some grumbling by the traditional universities. There has been particular alarm that the funds of £175 million or so now transferred from the universities' budget to the research councils to compensate grant-recipients for their overhead costs may be spread more widely. But fighting that battle must be unproductive, to say the best of it. Far

better to fight for the funds required to get the best out of the enlarged university system that has sprung into being — and to preserve as much as possible of its diversity. □

France changes guard?

The Mitterrand government seems to be nearing its end, but the president himself may soldier on.

THE general opinion in France that the present government will be defeated in the elections 17 days from now is probably correct. The call by senior figures in the ruling party for a new grouping of the centre-left is telling, as is the courtship of France's two green parties by all factions. Even if the president, M. François Mitterrand, decides to soldier on for part of the remainder of his second eight-year term, the chances are that he will then be even less in charge of events than during the short spell of cohabitation with the Chirac government of 1986. At best, it would be a sharp contrast with the heady election in 1981 when Mitterrand and his Socialist Party swept into power with an adventurous agenda of reform.

Much of that seems destined to endure. Mitterrand's socialism was never Marxism, although his first government was marked by a strong streak of syndicalism. His most dramatic innovation was the plan to increase public spending on research with the deliberate intention of making France more prosperous. For a year or so, budgets did actually increase by the 25 per cent a year promised. Although the accompanying programme of public ownership quickly caused a financial crisis and a change of course, the momentum was never entirely dissipated.

Not everything has turned out as intended, of course. The hope that France would become a giant in the computer business has been disappointed. And as elsewhere, too much of French industry remains over-closely tied to defence procurement. For better or worse, it is also true that there would not have been European civil aviation and aerospace industries without French pleading, even bullying. But there has also been a sustained wave of innovation in industries as different as chemicals and transport, notably on the railways. There has also been an improvement in the quality of higher education throughout France as well as a marked deepening of the education of graduate students. Those now searching for a recipe for wealth creation in Britain might do worse than take a leaf from Mitterrand's book.

Why, with this record, should Mitterrand's government be on the edge of extinction? Most of the explanation is political, but there are two respects in which the implications of a modernized France were never made clear to those affected. First, the logic that the creation of highly paid technical jobs makes farming less viable (or the cost of subsidies ruinous) seems not to have been explained to the rural communities of France. And France, as staunch a pillar of the European Communities as Germany, seems stubbornly to regard Europe as self-contained and potentially self-sufficient. Even if the opinion polls are right, not much of that is likely to change. □

ICI transfers most of its research to new Zeneca biosciences company

London. The British chemicals giant Imperial Chemical Industries (ICI) is to split its research activity in two following the separation of its bulk chemicals and bioscience activities into separate companies. Two-thirds of its total research budget, which last year was £720 million (US\$1 billion) — the highest of any company in Britain — will now be transferred to a new company, Zeneca Group, being set up to bring together ICI's interests in pharmaceuticals, agrochemicals and seeds. The name, which comes from the word "zenith", was changed from the originally proposed title ICI Bioscience.

The planned demerger, first proposed last summer and still awaiting formal endorsement by shareholders after last week's approval by the board of directors, represents one of the biggest changes in the company's history. ICI was formed in 1926 as a conglomerate of smaller chemical companies trying to achieve economies of scale in both research and production that were needed to compete internationally, in particular against Germany's IG Farben.

The reversal of that trend reflects a recognition of the different intensity of research and development in the two sectors. "The name of the game has to be focus", Sir Denys Henderson, ICI's chairman, said last week.

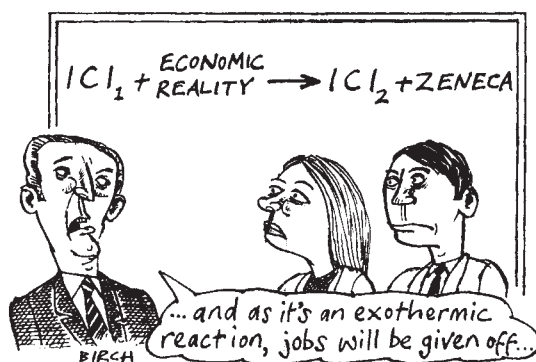
The division of research activities follows this logic. In the past, ICI built up large centralized research laboratories, based primarily at Runcorn in Cheshire, to do research that it hoped would eventually lead to new products or processes. Since the mid-1980s, however, research has been increasingly decentralized, and research teams linked directly to individual product lines.

The creation of Zeneca continues that pattern, with virtually no overlapping research between the two new companies. "The idea was to create a clear-cut separation in both research and technology", says Peter Doyle, formerly director of research and technology of ICI who will become executive director of Zeneca responsible for research, technology and manufacturing.

Exceptions will involve the services provided by the Central Toxicology Laboratory at Macclesfield and the Group Environmental Laboratory (to be known as the Brixham Environmental Laboratory). Both will become part of Zeneca, but will sign contracts with ICI to provide specialist services as a 'special customer'. Research and development in areas ranging from specialized plastics to chlorofluorocarbon substitutes will remain an important activity for

the "new ICI", which will have a research budget of about £250 million a year and a work force of 5,000.

The split recognizes the fact that the ICI's bioscience activities, which grew rapidly in the 1980s, are now large enough to survive on their own. Zeneca itself will



initially have sales of approximately £4 billion a year, and aims to be "a major force in the bioscience industry", according to its chief executive David Barnes.

The demerger will permit the growth of technologies based on new fields of science — in particular genetics and molecular biology — that some feel were neglected in the past in favour of the more traditional disci-

plines related to bulk chemical production. The impact of these new techniques, each based on what the company describes as a "common science base" underlying all Zeneca's activities, is expanding rapidly. For example, the development of new seed strains has been significantly enhanced by the introduction of genetic fingerprinting, which allows research workers to evaluate the effects of breeding techniques without a lengthy wait for genes to express themselves.

In order to encourage a more long-term focus, a "strategic research and development fund" of several million pounds has been set up to support grant applications from company scientists. However, any application for money from this fund still has to demonstrate how the research is expected to contribute directly to a specific product area.

Many ICI scientists regret the disappearance of the central laboratories. "For young scientists, joining ICI now

means that they do not have the freedom that they would have had in the corporate laboratory", says a former ICI chemist.

Doyle, however, believes that a demand for greater accountability of research reflects the times. "These days, exploitation and innovation are key themes, and we want to tie research more tightly to strategic work", he says.

David Dickson

Kessler stays, Healy goes

Washington. David Kessler has been asked to stay on as commissioner of the US Food and Drug Administration (FDA); on the same day (26 February), the Clinton administration accepted the resignation of Bernadine Healy as director of the US National Institutes of Health (NIH).

Kessler directs an agency with responsibility for the safety of foods (except meat and poultry) and cosmetics and the safety and efficacy of human and animal drugs and medical devices. Since taking up the post in late 1990, his biggest challenge has been to restore the reputation of an agency damaged by the discovery that several FDA officials had accepted money to speed up the review of certain generic drugs.

Kessler has cracked down on companies making unfounded commercial and health claims on food labels and has also tried to reduce the time needed to review therapies for patients with AIDS and other life-threatening diseases. He has also worked closely with Congress and the drug industry to

create a system of user fees that will pay for an additional 600 medical reviewers.

In contrast, Healy's two years at NIH have been marked by continuing tension between the agency and Congress as well as widespread unhappiness in the research community with her effort to draw up a strategic plan. Although she has won credit for rapidly expanding research on the health of women and minorities, she has been criticized for going along with the Bush administration's ban on fetal tissue transplantation research and its opposition to legislative changes to strengthen various initiatives.

Healy will leave on 30 June and will return to the Cleveland (Ohio) Clinic Foundation, from which she has been on leave. Her resignation, together with Walter Massey's departure from the National Science Foundation at the end of the month, gives the new president a chance to put his stamp on the agencies that fund most of the country's academic research.

Diane Gershon & Jeffrey Mervis

Taiwanese panel rejects spending \$64 million on SSC

Tokyo & Washington. In a possible setback for the US Superconducting Super Collider (SSC), Taiwan's leading science policy-making body last week voted not to participate in building one of the main detectors of the collider. But the decision by the National Science Council of Taiwan must be reviewed by the Taiwan cabinet (executive yuan), which has just been reformed following the appointment of a new premier.

On 22 February, a review panel of the council voted 12 to 4 against a proposal to spend US\$64 million in Taiwan on building the central tracking system for the \$530-million GEM (gamma rays-electrons-muons) detector, one of two planned for the collider. About \$50 million would be spent on the design, construction and assembling of the tracker and the rest on a scientific exchange programme and the purchase of equipment. The tracker would be built at Taiwan's Industrial Technology Research Institute (ITRI).

The US government is providing only about half of what is needed to build the two detectors, GEM and SDC (Solenoidal Detector Collaboration), so outside contributions are essential to their success. The SDC will cost an estimated \$610 million.

Proponents believe that participation in GEM would benefit high-energy physics in Taiwan, advance ITRI's goals of developing electronic packaging, composite materials, integrated optoelectronic circuits and communication systems and teach the institute how to integrate a complete high-

technology system. Critics say that there is little new technology to be learned, that Taiwan has no infrastructure and very little experience in high energy physics and that the investment is risky because SSC officials may decide to cancel or delay GEM to save money (see below).

The council vote came one day before Taiwan's president, T.H. Lee, appointed a new premier, Chan Lien, to head the executive yuan, which must approve the council's decision. And Lien's choice to be chairman of the council, N. Kuo, supports Taiwan's participation in the SSC.

SSC officials and scientists involved in the collaboration say that they expect the new cabinet to overrule the council and reaffirm a cooperative agreement signed last November between the SSC laboratory and the Taiwanese government. The council has "flipfopped" on the SSC issue in the past, says Shih Chang Lee, deputy director of the Institute of Physics of Academia Sinica, which is one of the partners in the collaboration. Rather than either accepting or rejecting the council's recommendation, the cabinet could ask the council to reconsider the matter.

Work on the central tracking system will continue regardless of what the Taiwanese decide, according to the head of the US team, Charles Baltay of Yale University. "The collaboration has been under way for a year," he says, "and the only issue is whether they will put up big or little bucks".

David Swinbanks & Jeffrey Mervis

UK universities get funding on basis of ranking

London. British universities, old and 'new', are feeling the effects of a research assessment exercise carried out last year, with some reaping the rewards of a good rating and others experiencing a loss of funding for the next academic year.

Both Cambridge and Oxford, for example, which topped the league in terms of the number of high-scoring research departments, will enjoy an increase of more than 11 per cent in total public funding, including a 15 per cent increase in support for research (see table below). Those 'new' universities — previously known as poly-

England's Top Ten (in £ million)

University of Oxford	40.63
University of Cambridge	40.04
University of Manchester	28.11
University College, London	25.68
Imperial College, London	23.56
University of Birmingham	21.22
University of Leeds	21.07
University of Liverpool	18.35
University of Nottingham	17.87
Kings College, London	14.65

technics — that did well in the assessment exercise will share £36 million (US\$50 million) for research and an additional sum for "the development of research potential".

In contrast, 'old' universities that did relatively badly in the assessment exercise, such as Aston in Birmingham, Bradford and Salford, will see their public funding reduced by one per cent.

The grants next year to 200 higher and further education institutions in England were announced last week by the Higher Education Funding Council for England (HEFCE), which has absorbed the funding responsibilities of the Universities Funding Council and the Polytechnics and Colleges Funding Council. (Separate figures will be announced later for Scotland and Wales). Overall, funding allocated for research in these institutions through the HEFCE will increase by 9 per cent, to £618 million.

The money has been distributed according to a three-part formula. Just under 95 per cent of the total — £585 million — has been allocated on the basis of their ratings (see *Nature* 360, 700; 1993). A further £20.5 million was distributed to universities on the basis of their success in obtaining outside contracts. The remaining £12.5 million has been distributed to 55 institutions considered capable of delivering high-quality research.

David Dickson

SSC report finds overruns

Washington. A new analysis for the US Congress of the Superconducting Super Collider (SSC) has found serious cost overruns and poor management practices in the \$10-billion project. The results are disputed by the Department of Energy, which says that the report relied on outdated and inaccurate information, but it seems certain to prove useful to congressional opponents in their annual fight to terminate the programme.

The General Accounting Office (GAO) last month reported to the Committee on Science, Space and Technology of the US House of Representatives that the cost of conventional construction may exceed the \$1.25 billion estimate by half on the basis of what has been spent on the first 10 per cent of the work. The cost of making the 8,000 superconducting dipole magnets that bend the two opposing proton beams was similarly running 25 per cent above projections, the GAO reported, and SSC officials had not included the cost of storage if the magnets could not be installed promptly in the 54-mile-long tunnel being dug in Waxahachie, Texas. The problem, according to the GAO, is that the consortium of universities managing the project "still does not have a system in place that would allow it to objectively monitor the project's cost and schedule".

In a letter of 24 February to US Representative George Brown, chairman of the science committee, Energy Secretary Hazel O'Leary says that the GAO ignored relevant information and distorted spending patterns in reaching its conclusions. Although O'Leary insists that the project is on schedule for completion in 1999 for \$8.25 billion, she admits that a recent decision by President Bill Clinton to extend the programme by four years will raise the cost.

J.M.

Scientists criticize DOE work on nuclear repository site

Washington. A group of scientists who have reviewed plans for studying a proposed nuclear waste storage site in Nevada say that US government project managers have failed to follow the recommendations of a report issued last April by the National Academy of Sciences, with science taking a back seat to engineering and budgetary considerations. The scientists this week sent a letter expressing their concerns to the US Department of Energy (DOE), which manages the Yucca Mountain site survey.

The site has been controversial ever since it was chosen in 1987 as the prime candidate

just north of the site to characterize the regional hydrogeology better.

But DOE has chosen to "brush off" these and other suggestions, says George Thompson of Stanford University, vice chairman of the panel. In a 39-page letter sent in October to the academy, DOE said that exploring the gradient was worthwhile but probably would not be done for three to five years and that the deep drilling programme, if done at all, may include fewer holes than the panel recommended.

The response so displeased the panel members that they decided on the unusual step of a follow-up letter, even though the panel has been officially disbanded. All eight of the former panel members contacted about the letter agreed to sign, expressing frustration at what John Bredehoeft of the US Geological Survey (USGS) says is the DOE's attitude of "treating this thing as an engineering construction project instead of a broad-based scientific investigation".

Although DOE agrees with many of the panel's recommendations, says project manager Carl Gertz, its first step is building a huge ramp that will allow scientists access to the interior.

While he is managing that engineering task, Gertz must also deal with pressure from Congress to go faster and spend less, with opposition from Nevada state officials and with public scepticism about the long-term safety of the repository. The government is required by 1998 to begin storing nuclear waste now piling up at power plants, so temporary sites will have to be used until a permanent repository is ready.

Gertz says that a chief scientist is not necessary because he already receives enough advice from researchers working on the project, including more than 200 from USGS. But investigators working at Yucca Mountain complain that most of the current budget is spent on engineers and administrators, who routinely ignore their advice and needs. The decision rests with the new DOE management team led by Energy Secretary Hazel O'Leary.

The members of the academy panel who signed the letter are not claiming that Yucca Mountain is unsuitable for storing nuclear waste. But they believe that the hydrogeology of the mountain must be better understood before DOE decides whether the repository is likely to remain safe and dry for at least 10,000 years.

Tony Reichhardt

US judge throws out laboratory rules for dogs, primates

Washington. A US federal judge has invalidated the system to regulate dogs and nonhuman primates used in research, in part relying on an ambiguous phrase in a 1985 law amending the Animal Welfare Act that has long bedevilled scientists. The government's dependence on veterinarians and institutional review committees has produced regulations that are "arbitrary and capricious", said the judge, who ordered the government to issue new regulations as quickly as possible. An appeal is considered likely.

District Court Judge Charles Richey ruled on 25 February that the US Department of Agriculture (USDA) and other federal agencies have failed to set minimum standards for the care and treatment of research animals, in particular criteria for exercising dogs and for ensuring the "psychological well-being" of primates. The latter is a phrase intended to improve conditions for research animals that was inserted by then Senator John Melcher into a 1985 amendment to the animal welfare laws. It has spawned considerable research, none of it conclusive, aimed at clarifying the conditions under which various species should be housed.

The suit was filed in May 1991 by animal rights groups after the government issued final regulations implementing the legislative changes. An earlier set of regulations was substantially revised after institutions complained about the cost of larger cages and more personal care.

"What Judge Richey has said is that USDA has to be the author of any regulation and that its delegation to the research industry of that power is illegal", says Valerie Stanley, the lawyer for the plaintiffs. "He also said that the rules must establish minimum standards, and that the industry's argument that there are too many exceptions doesn't hold water."

Last year, Richey ruled in favour of the same plaintiffs in a suit that challenged the USDA's exclusion of rats, mice and birds from the same law. In that case, against which the government has appealed, Richey said that there was no reason to omit the millions of rodents and birds used in research from its routine inspections and from guidelines for their proper care.

Research institutions believe that compliance will force them to spend millions of dollars on care that has not been shown to be beneficial to the animals. "The rules were meant to be a little vague", says Richard Traystman of Johns Hopkins University Medical School, "because we don't know, for example, what psychological well-being is for a monkey. The judge wants some precise answers, but they don't exist."

Jeffrey Mervis



DOE is taking a \$6-billion look at Yucca Mountain.

for a proposed underground repository for dumping nuclear waste from civilian US power plants. The mountain was selected for its geological stability and for the lack of rainfall in the region, which reduces the risk of radioactive contaminants leaching into groundwater. The site characterization studies, which will take another eight years, are expected to cost \$6.3 billion.

The National Academy of Sciences became involved in the controversy after a DOE staff hydrologist, Jerry Szymanski, claimed to have found evidence that groundwater under the mountain had risen high enough in the geological past to suggest that the repository might become flooded in the future. After two years of looking into the claim, the academy panel dismissed that worry — as have most independent scientists who have looked at Szymanski's evidence — but criticized other aspects of the project.

The panel report cited "a significant lack of communication among project scientists in different disciplines" and said that a chief scientist is needed. The report also recommended drilling deep into a palaeozoic aquifer under the area and taking core samples from a steep gradient lying

Interferon spurs growth of Japanese biotechnology

Tokyo. Interferon, once thought to be a possible miracle cure for cancer, has emerged as an important element in the growth of Japan's biotechnology industry as a treatment for a type of viral hepatitis prevalent in Japan.

Sales of interferon soared from ¥30 billion (US\$250 million) in 1991 to ¥150 billion last year after the drug was approved by the Ministry of Health and Welfare for treatment of chronic hepatitis C. This year, sales are expected to exceed ¥200 billion, about a third of Japan's total biotechnology market, according to the newsletter *Nikkei Biotechnology*.

In fact, its accelerated growth has led the Ministry of Finance to call for restraint in its use in fear that it may become a drain on the national health insurance system. A growing number of reports of serious side effects may also limit its market.

In the early 1980s, Japan's fledgling biotechnology industry invested heavily in interferon because it was thought to be a possible cure for cancer. Those hopes proved to be largely unfounded, although the drug has been approved for treatment of a few limited types of cancer.

Sumitomo Pharmaceuticals holds the biggest share of the market — more than a third — because of its large production capacity. Sumitomo licensed cell culture technology from Wellcome plc in Britain and built one of the largest culture plants in the world with three bioreactors of 8,000 litres capacity each. "At the time many people thought they had made a mistake", says Mitsuru Miyata, editor of *Nikkei Biotechnology*, but Sumitomo's investment has allowed it to respond quickly to increased demand.

Two competitors, Daichi Pharmaceuti-

cal and Toray Pharmaceutical, have their own cell-culture technology and two pairs of companies — Takeda Pharmaceutical and Nippon Roche, and Yamanouchi Pharmaceutical and Schering-Plough — apply recombinant DNA technology licensed from US companies. Hayashibara is awaiting approval for its novel technique of mass-producing interferon in cancer cells in millions of mice. The demand for interferon is so great that the National Institute of Health, in an unprecedented move, last year accelerated its routine testing of the safety of production lots of the drug.

Japan has about 1.5 million carriers of the hepatitis C virus, a far larger proportion of the population than in the United States and Europe. Tens of thousands with evidence of active chronic hepatitis in liver biopsies are allowed interferon treatment.

The surge in national health insurance claims for interferon treatment, which now account for about 1 per cent of Japan's total medical bills, has caught the attention of the Ministry of Finance. And in January the Ministry of Health and Welfare, under pressure from the finance ministry, issued a directive to prefectural governments calling for restraint in its use. But it can be argued that conventional treatment of hepatitis C, a disease that often ends in development of liver cancer, would in the long run cost more.

There are also side-effects, including influenza-like symptoms and, in some cases, pneumonia, possibly linked to its use in combination with traditional Chinese medicine.

Nevertheless, it seems that hospitals cannot get enough of the drug, and its use is expected to increase several-fold in the coming fiscal year which begins on 1 April.

David Swinbanks

Vaccination may be a culprit

Tokyo. The prevalence of hepatitis C in Japan may in part be an unfortunate product of modern medicine.

The original cause of the high incidence of hepatitis C in Japan and other neighbouring Asian nations is not understood. However, it is believed that the common practice of reusing needles in mass vaccination programmes carried out several decades ago in Japanese schools helped to spread the virus. Several lawsuits have been filed by patients blaming the vaccination programmes for their infection but it is impossible to trace the source of infection with certainty.

Another contributing factor was the absence of routine screening for the virus in the blood supply until detection kits for the hepatitis C virus were developed following characterization of the virus in 1988. As a result, about 10 per cent of patients receiving blood transfusions in Japan were infected with the virus because of the high incidence of hepatitis C among blood donors. **D.S.**

Tokyo University names new head

Tokyo. Faculty members of Tokyo University, Japan's leading national university, last week elected a president to replace Akito Arima, a leading force for reform of Japan's universities.

Arima's successor is Hiroyuki Yoshikawa, a former dean of the faculty of engineering

and now a special adviser to Arima, whose four-year term ends on 31 March. Yoshikawa is known for his work on the Intelligent Manufacturing Systems (IMS) project, which is intended to combine the industrial forces of



Hiroyuki Yoshikawa

Japan, Europe, North America and Australia to develop a fully automated manufacturing system (see *Nature* **355**, 755; 1992).

But Yoshikawa will find it hard to match the achievements of Arima, who has not only helped universities to get substantial new government funding for renovation and for research but has also initiated the first external review of a university department (see *Nature* **360**, 403; 1992). Yoshikawa must decide whether to extend the review process to other departments.

David Swinbanks

Pricing system encourages sales

Tokyo. Apart from responding to patient demand, Japanese hospitals have a strong financial incentive for prescribing expensive interferon treatment (see above).

In Japan, hospitals buy and sell their own drugs. Although the Ministry of Health and Welfare sets official prices, hospitals routinely buy drugs at a greatly discounted price from pharmaceutical companies. With demand for interferon so strong, drug companies do not have to offer large discounts. But as a course of treatment with interferon typically costs several million yen (several tens of thousands of dollars) for a patient with hepatitis C, even a small discount can mean a sizeable profit. One result of the system is the highest per-capita consumption of drugs in the world.

Apart from cost, overprescription of expensive drugs has serious medical consequences. Japanese doctors routinely give large doses of expensive antibiotics to patients after minor operations even if they show no signs of infection. As a result, many of Japan's hospitals are now infected with resistant strains of bacteria that can be lethal to patients with weak immune systems.

The Ministry of Health and Welfare has for years tried to separate the prescription and selling of drugs by urging hospitals to use outside pharmacies, and it is gradually reducing the maximum discounts that drug companies can offer.

D.S.

Rwandan war threatens gorilla research project

Washington. A civil war in Rwanda has forced gorilla researchers to flee the country and threatens to disrupt a 25-year record of observations begun by primatologist Dian Fossey.

Some 300 mountain gorillas, nearly half the global population, live in the park, according to Dieter Steklis, director of the Karisoke centre, and the death of even a few females could have a major impact on the population. The centre, sponsored by the Dian Fossey Gorilla Fund, studies two groups of 36 and 18 animals and tracks three other groups that are visited by tourists. (The Morris Animal Foundation's veterinary centre monitors the health of the entire population.)

Ruth Keating, DRG

Steklis is also concerned that the trust of the two research gorilla groups in humans, which takes years to develop, may be shattered by the events of the past few weeks. "We're talking

about animals Dian knew personally", Steklis says about the work of Fossey, who was killed in 1985. The loss of such individuals and their life-long records would be a scientific tragedy, he says.

The Rwandese who fled the advancing soldiers reported finding a great deal of gorilla blood as they crossed the trail of one of the gorilla groups, says Steklis, adding that the workers are trying to return to check on the gorillas. Steklis says that the animals are endangered not just by gunfire but also by the illegal hunting of antelopes by both civilians and soldiers, an activity that increases sharply when there are no patrols.

Jenna Roberts

IMAGE
UNAVAILABLE
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REASONS

The Karisoke Research Center in Rwanda.

The ransacking on 18 February of the Karisoke Research Centre in the Volcano National Park and the Volcano Veterinary Centre by soldiers of the Rwandese Patriotic Front followed ten days of violence in and around the previously protected animal sanctuary. Five foreigners (two British, one German and two US scientists) were evacuated safely after being trapped for five days at the centre, and five days later 13 Rwandese workers fled before the advancing soldiers, who shot out windows and looted the single-storey buildings. The civil war began in 1990, but both the rebels and the government had pledged to keep fighting away from the area.

NSF rethinks its proposal to revise page-charge rules

Washington. A proposal by the US National Science Foundation (NSF) to allow its grants to cover page charges in commercial journals has tapped into a vein of complaints about the pricing of journals and has opened a debate on ways to help libraries defray soaring costs.

The NSF proposal, which has attracted more than 40 responses since it appeared in the *Federal Register* last December, was a modest attempt to restore balance to a 60-year-old system that allows NSF grants to be spent on page charges in journals published only by nonprofit organizations such as professional societies. The rule was implemented to help societies survive the depression of the 1930s by subsidizing a part of their publishing costs, and page charges remain an important source of revenue for some scientific organizations.

Last autumn, a publishing company that does not now make page charges questioned the fairness of the rule and suggested that NSF should stop paying all page charges and instead find a way to give the money directly to academic libraries. But NSF chose to correct the perceived unfairness by making no distinction between commercial and non-profit organizations, bringing it in line with the National Institutes of Health.

"We intended to find a vehicle for the NSF to reallocate the money, and instead they are addressing a small section of the issue and precluding a change to abolish page charge payment", says Leslie Parks of the Susan Davis Company, a consulting firm hired by the publishing house.

Most commercial publishers do not make page charges. Four commercial publishers supporting the change emphasized in letters to NSF that they did not plan to adopt page charges but that reforms are needed in the way in which libraries are funded.

The American Geophysical Union (AGU), whose journals are subsidized by page charges, strongly objects to the proposed change, saying that the new policy would encourage commercial publishers to institute page charges, spreading the money over a larger pool and reducing revenues for journals published by non-profit organizations. But William Kaula, a geology professor at the University of California, Los Angeles, says that incentives at present actually favour commercial publishers by allowing scientists to use their money for other things and that page charges in commercial journals may encourage researchers to submit papers to AGU publications.

NSF has decided to take more time to digest the unexpectedly large volume of responses and has set no deadline for a decision.

Jenna Roberts

UK engineering curriculum revised

London. The Science and Engineering Research Council (SERC) is planning a major shift in the training of postgraduate engineers that will offer courses and doctorate schemes with much greater practical content and industrial involvement.

A review carried out by the education and training committee of the council's engineering board, and published in London on 1 March, supports moving away from the present three-year university-based PhD taken by most postgraduate engineering research students. The committee also recommends that funding for one-year MSc courses be reduced by 30 per cent and that the money be used for modular postgraduate courses for those already working. It also proposes training vouchers, worth £400 each,

for SERC-funded PhD students for short courses aimed at broadening their range of technical and social skills.

"We must maintain a proportion of engineering graduates in traditional postgraduate work", says James Powell, professor of engineering at Brunel University and chairman of the committee. "But we must shift the balance more towards the engineering side if we are to get the proper integration of theory and practice."

The report recommends that the SERC should aim to have 30 per cent of postgraduate students studying for the new engineering doctorate. Money is a limiting factor, according to Powell, who says that "we would be more bullish if the government were to give us more support". **David Dickson**

South Africa endorses nonracial schools and increases subsidies

Cape Town. The South African government has agreed to a single nonracial system of education as well as an increase in subsidies to universities for training more minority students in science and engineering.

Last month, the ruling National Party and the African National Congress (ANC) both released their new education policies. The ANC's "National Education Policy Investigation" is more vague than the "Education Renewal Strategy" (ERS) proposed by government, but all sides agree on the principle of one system.

The university subsidies are awarded according to a formula that takes into account undergraduate and postgraduate student numbers as well as their respective success rates. It also gives greater weight to those enrolled in the natural sciences, including science, engineering and medicine. But for the past five years the government has abandoned the formula, fixing subsidies at a straight percentage increase on the 1988 subsidy award, which reflects 1986 enrolment numbers.

The ERS recommends that the government revert immediately to the formula and provide 70 per cent of the subsidy due to the universities. This year, the government has given universities 65 per cent of their entitlement with the proviso that none should receive less in nominal terms than last year.

The restoration of the formula will redress differential rates of growth in the universities since 1986. With inflation running at 10 per cent, a handful will receive a cut in real terms of a similar magnitude. But most of the established universities have grown modestly during the past five years and some, like the University of the Western

Cape and the University of the North, have doubled and tripled respectively in size. In addition, the formula has been modified by altering the weighting from 1.6:1 to 2.5:1 in favour of students in the natural sciences.

However, it is not as yet clear whether this will have the desired effect of increasing enrolment in these fields. The ANC document suggests a more radical solution: a centrally planned system in which the state would subsidize a fixed number of places in each field and then distribute them nationally.

A single education minister will be appointed by 1 April and given one year to integrate the existing racially constituted education departments into new regional executive departments. (There is at present one minister each for African, Coloured and Asian education plus Minister of National Education Piet Marais, who is responsible for both white education and policy coordination.) The ERS envisages three types of schools in the new dispensation: private schools (which nevertheless receive a large government subsidy), state-aided schools (for which the state pays salaries but not other expenses) and state schools (for which the state foots the entire bill).

The proportion of the total education budget allocated to postsecondary education has remained steady, with universities and technical schools getting a combined 15 per cent of the overall budget and teacher-training colleges receiving an additional 5 per cent. Although this year's spending figures will not be released until the budget is announced next month, Marais hinted at an increase in the overall education allocation during a press briefing last week.

Michael Cherry

NEWS IN BRIEF

Munich. Germany's new research minister, Matthias Wissmann, has won a battle for more money to strengthen research in eastern Germany. The government last week added DM50 million (US\$30 million) to an annual budget of DM330 million for applied research projects and promised to increase next year's budget by DM50 million. Industry has been promised an equal amount this year as part of a package to improve the flagging economy in the former East Germany. **A.A.**

Washington. Ashton Carter of Harvard University, a vocal critic of the original claim that the Strategic Defense Initiative (SDI) could protect the United States against an all-out Soviet attack, is expected to be put in charge of that and other programmes at the Defense Department. Carter joined many scientists in the mid-1980s in arguing that a directed-energy weapons system was not feasible. In recent years the debate has shifted to whether any type of SDI-type defence system is worth the cost (this year SDI will receive \$3.5 billion, and more than \$20 billion has been spent on it since 1983). Carter, as an assistant secretary of defence for policy, will also handle strategic issues such as nuclear nonproliferation and the dismantling of Soviet warheads. **J.M.**

Munich. More than 5,000 biochemical researchers have signed a resolution calling on German scientists who have "friendly relationships and close personal contacts" with foreign colleagues to support and protect foreigners in Germany. "There is no place for discrimination and hatred as part of a peaceful Germany", the resolution states. Recent violent attacks on foreigners have raised questions about the wisdom of holding the IXth International Conference on AIDS in Berlin in June. But its chairman, Karl-Otto Habermehl, says that a proposal to hold the congress outside Germany is unrealistic and not an answer to the problem. The attacks have not so far had a significant effect on applications, and some 15,000 people are expected to attend the meeting. **A.A.**

Munich & London. Antonio Ruberti, the new research commissioner for the European Communities, is showing clear signs of respecting the advice of scientists. Since taking up his post in January, Ruberti has appointed as personal advisers two Nobel prizewinners — Belgian chemist Ilya Prigogine and Italian particle physicist Carlo Rubbia — and French biologist François Gros, a former science adviser to the French president, François Mitterrand. Speaking last week at a symposium in Oxford on science and society, Ruberti said that he wanted "a closer relationship with the world of science" and to open up new areas of cooperation. **A.A. & D.D.**

London meetings on British science

On Friday 19 March, there will be a meeting at the Royal Society, 6 Carlton House Terrace, London SW1, to discuss proposals for the forthcoming White Paper on the organization of British science. The speakers will be Sir Eric Ash (Rector, Imperial College London), Professor Michael Brady (University of Oxford), Dr Dai Rees (Secretary, Medical Research Council) and Sir Mark Richmond (Chairman, Science and Engineering Research Council); the meeting will start at 9.30 a.m. and end at 4.00 p.m.

Admission to the meeting will be by ticket, free of charge, obtainable from Mary Sheehan, Nature, 4 Little Essex Street, London WC2R 3LF. Coffee and tea will be provided.

There will also be a sandwich lunch for those who want it, **at a cost of £5:** please send a cheque made out to *Nature* with your ticket application. Tickets for both the meeting and lunch will be sent out during the second week of March.

* **On Thursday 18 March,** Save British Science is holding a meeting called "Waldegrave meets the scientists" at 12.30 p.m. in the Great Hall, Sheffield Building, Imperial College, London SW 7. The Rt Hon. William Waldegrave, MP, Chancellor of the Duchy of Lancaster and Minister of Public Service and Science, will speak and will answer questions. Entry free. Seats may be reserved by application to Save British Science, PO Box 241, Oxford OX1 3QQ (tel. 0865 273407, fax 511370).

UK government research

SIR — I should like to add to your Commentary "How will Britain run its science now?" (*Nature* 361, 581–584; 1993) one additional point that I think is worth making about government research. It is important to distinguish research that is undertaken by departments in order to fulfil their prime functions (for example the Ministry of Defence, the Department of Health, Transport and so on), from research that is sponsored for the broader national good by departments such as the Department for Education, the Department of Trade and Industry and the Office of Science and Technology, the results of that research being primarily of interest to others.

Departments of the first kind may be expected — like major international research-dependent companies — to do no more research than is necessary to fulfil their missions. As an example, the Ministry of Defence research programme is focused on government defence objectives and is, therefore, weighed against other priorities in the defence budget. The aim is to find the right balance between research and other elements such as personnel, equipment, training and so on, and the implication is that if the research spend were different from what is finally agreed, the country would get less good value for money in defence. In consequence, the scale of research spend of 'research-user departments' can be expected to reflect government objectives for those departments,

rather than to comprise any particular proportion of the national research spend.

These issues are, however, separate from that of how the country should best go about deriving as much secondary benefit as possible from research that the 'user departments' carry out, whether through collaboration, coordination of related work of different sponsors, or disseminating results outside government. UK government departments and universities alike have had initiatives to promote commercial exploitation of work from their laboratories; few, however, are totally content with what they have achieved and we have seen good ideas unexploited, or exploited abroad — liquid crystals that find a hundred-and-one day-to-day applications were developed at the former Royal Signals and Radar Research Establishment at Malvern but turned into a commercial product by the Japanese. It is in this area that we have to find some different way of managing our affairs.

Ronald Oxburgh
(Chief Scientific Adviser)
Ministry of Defence,
Main Building,
Whitehall,
London SW1A 2HB, UK

SIR — Although much of the support for science, from the public or private sectors, must clearly be for science with an application in mind, it is essential that there should be adequate funds (wholly

or largely provided by government) for curiosity-directed research. This is a cultural requirement, just as public support for the arts is. The amount provided needs to be secure, and the only objective in its disbursement should be to help create the best science possible.

Wherever given funds are employed to create science, whether basic or applied, the division of funds between supporting scientists on the one hand and equipping them on the other must be governed by the need to get the best science for the sums supplied. Inability to fund good proposals is a clear sign that, for the money available in the field in question, there are too many scientists working in it. Fruitlessly chasing funds is an inefficient use of scientists' time.

As there is good reason to suppose that in many fields the cost of properly supporting a scientist with equipment is growing faster than the funds likely to be available, it follows that in any such field the number of working scientists should diminish year by year. It may be difficult to attain the optimum numbers in a field, but the objective of matching the numbers to funds available should be kept firmly in mind. Failure to do so is unfair to the provider of funds, and bad for science.

Where researchers are supported because of the prospective benefit to British industry, it is essential to ensure that companies are in a position to exploit a research success. A repetition of the liquid crystal fiasco would be hard to accept.

It is important to have a system that can take account of the basic differences between applied sciences such as engineering and the fundamental sciences such as chemistry and physics. For many of the ablest pure-science undergraduates, research in their field is the principal ambition, whereas many of the ablest engineering undergraduates aim to practise engineering as soon as possible. Thus whereas in science one can expect to fill a research studentship with the highest level of graduate, this cannot be taken for granted in engineering.

The weakness of the connection between defence and civil research and development is particularly noticeable in Britain, as the recent POST report made clear. One would hope that at least a beginning can be made on bridging this gap.

The forthcoming White Paper will address questions of organization. It is important that the bureaucracy should be aware of the needs of the working researchers. In particular, those disbursing funds must visit the laboratories where the work is actually being done.

Hermann Bondi
Churchill College,
Cambridge CB3 0DS, UK

Countering the calories

SIR — With his usual perspicuity, Daedalus has pointed out (*Nature* 360, 112; 1992) the way to solving the relatively affluent's dilemma of I-don't-want-to-be-fat but I-do-want-to-eat.

He neglected to mention — probably for commercial considerations — the real reasons why dinitrophenol is the compound his researchers are almost certainly derivatizing right now, seeking the permutation that retains all its desirable traits while eliminating its undesirable ones.

The billion-dollar diet-aid potential of dinitrophenol derivatives (DNDs), of course, lies in the parent compound's biochemical role as a decoupler of oxidative phosphorylation. The mitochondria, quite sensibly evolutionarily speaking, tightly link the burning of food to the creation of ATP and, if this is not used up, the furnace shuts down and the food gets shunted off to the fat reserves.

Carefully tailored for organ and metabolic specificity, the DNDs will circumvent this now-disadvantageous coupling

and allow the Krebs' cycle to spin uselessly, converting unwanted calories to heat and carbon dioxide.

The commercial potential of DND rivals that of sugar and fat. It could, for instance, be premixed into fast-foods, dramatically reducing their calorific impact and increasing their consumerability. The cultural impact would also be sublime: the potential of sprinkling a meal of 2,000 calories down to just 15 would quickly clear restaurants of dinky starvation rations and reawaken an interest in the butter-saturated cooking of a bygone era.

I await the commercial introduction of DND with the interest only to be expected of a 15-pound overweight chap who cannot wait to resign from the self-imposed torture and tedium of his health club.

Richard L. Lewis
4 West 43rd Street,
Apt 517,
New York,
New York 10036, USA

The Lords fall down

SIR — I fear that the recently published report (see *Nature* 361, 481; 1993) on the Faraday programme falls short of the high quality we have come to expect from the House of Lords Select Committee on Science and Technology, which has missed a significant opportunity to influence national thinking on innovation. I could more readily accept the committee's rejection of the proposals of this centre's working group if the committee had offered some new ideas to improve the innovative capacity of small and medium sized companies rather than attempting to maintain the status quo.

Any survey of this type depends, of course, on the sample and the universe from which it is drawn. Industry represented just 15 per cent of the sample and half of these companies were from the chemical and pharmaceutical industries, sectors which we clearly stated in our reports and our evidence were unlikely to find merit in the proposals. In none of the working group's reports are new buildings proposed and we made it abundantly clear that *any* organization could be considered, including higher education institutes and industry laboratories, *provided* they satisfied the criteria, which are extensive interaction with industry, an established industrial customer base and an international reputation, and a track record in technology development and transfer.

The Faraday programme is primarily intended to improve the performance of medium-sized companies employing fewer than 500 people. I do not doubt that larger enterprises would benefit, but for the committee to interview only multinationals was a mistake. The Department of Trade and Industry (DTI) has conducted a similar review of attitudes amongst industrialists, including SMEs (small and medium-sized establishments), and I have a great deal more confidence in their validity.

The main categories from which the sample is drawn were government and institutes of higher education, although I note that the committee failed to interview any of the former polytechnics. Given that there was little prospect of new money, I am not surprised that government officials were noncommittal and the academics afraid of seeing their funding reduced still further.

Throughout the report there is little mention of technology development, the very essence of the Faraday concept. There appears to be no appreciation that technology is not the same as science and that development is substantially different from research. The development of generic or enabling technologies for industry is not the prime objective of

universities nor of any of the schemes mentioned in the report. LINK comes closest but I still believe that, for many medium companies, the prospect of dealing with a university is too daunting.

The report refers to a culture change within universities. I accept that they are beginning to appreciate their role in improving the competitiveness of industry, but many academics rightly perceive a conflict between maintaining a high research profile and extensive interaction with industry. The report suggests the large number of university-based collaborations bears testimony to this culture change. Relative to the scale of the challenge, I do not believe numbers are large and research clubs and part-time PhDs are of little consequence.

The select committee's report was published at a critical time, with the Office of Science and Technology and the DTI undertaking fundamental reviews and industry under pressure to create wealth and rescue the economy. The old ways have failed. My group identified a way out of the impasse and the debate has moved on. The Faraday principles have been widely endorsed, not least by the Secretary of State for Trade and Industry in his evidence to the committee. The DTI is looking to remove barriers to the participation of institutions intermediate between industry and the science base in various schemes.

Meanwhile, universities and technology development organizations throughout Britain are developing new relationships, incorporating the principles into new partnerships and seizing the opportunity to serve industry in imaginative and effective ways. The select committee has missed the opportunity to assist this process. As the provost of University College London said in evidence, "part of the British disease is to do nothing whilst considering the options".

John Fairclough
(Chairman, Working Group on Innovation)
Centre for Exploitation

*of Science and Technology,
5 Berners Road, London N1 0PW, UK*

Working for a test-ban treaty

SIR — Lord Zuckerman's article (*Nature* 361, 392–396; 1993) is a timely reminder that Britain should again be working for an early Comprehensive Test Ban (CTB) Treaty. It may be helpful if I clarify one point in the leading article in the same issue (page 381).

Referring to the CTB negotiations of

1977–80, which incidentally covered all but the first six months of President Carter's term, you say "But that treaty, which would have been signed by Britain, the Soviet Union and the United States, would not have met the present need". My own belief (as deputy leader and then leader of the UK delegation to the CTB negotiations in Geneva from 1977 to 1980), and that of former US negotiators I have spoken to this year, is that the main features of the treaty we were negotiating and drafting would meet the present need very well. We always made it clear that, once the three negotiating powers had agreed on a draft, it would go to the Conference on Disarmament and thence to the UN General Assembly for approval. It would then be open for signature by *all* states.

The main difference now is that the other two declared nuclear powers acceded to the Non-Proliferation Treaty in 1992 and could therefore logically join the original three in negotiating a CTB. There seems every prospect that France will wish to do so, and no doubt China would be welcome to join at any stage.

The important thing is to avoid providing excuses for still further delay in achieving a treaty to which Britain has been committed for some 30 years. The latest excuses tend to emphasize "safety", about which Zuckerman makes some telling comments. His doubts are reinforced by a recent statement by a number of eminent US nuclear scientists that "the advantages of an immediate mutual moratorium and of a CTB outweigh, in our judgement, any perceived benefits of further tests *for any reason*" (my emphasis).

In my judgement, it need not take many months to complete a treaty that would, in your words, be "genuinely comprehensive". This would demonstrate at last that the nuclear powers are prepared to accept restraints on their own nuclear weapon programmes and so greatly improve the case for preventing the further spread of such weapons. The main requirement now is not a technical one — it is the political will of the countries most concerned, and that includes Britain.

J. C. Edmonds
*North Lodge,
Sonning, Berkshire RG4 0ST, UK*

SIR — In the picture on page 395 of Yeltsin and Bush signing START 2 in Moscow, Yeltsin holds the pen in his left hand; Bush holds the pen in his right with the typical left-hander's arm curl. No wonder it looks odd: the picture is reversed.

David L. Waggoner
*1021 Arlington Blvd, Apt 641,
Arlington, Virginia 22209–2228, USA*

Iceman in the cold light of day

Paul G. Bahn and Katharine Everett

The discovery of a Neolithic corpse in an alpine glacier in 1991 attracted widespread attention. What has happened in the eighteen months since then reflects badly on European science.

As the first official reports¹⁻³ and popular accounts⁴ of the discovery of a prehistoric 'iceman' from the Alps began to appear, so did doubts and queries about the find and its circumstances start to surface⁵. The mummified corpse, popularly called 'Similaun man' or 'Oetzi', was found on 19 September 1991. It was first spotted, trapped in ice at 3,200 m altitude near a well-trodden walkers' route, by German tourists. With it were leather, grass, flint and wood artefacts.

The body and the other finds were not removed for 4 days, during which time at least 22 people, including the noted mountaineer Reinhold Messner, visited the site; some had tried to free the corpse from the ice. At this point some suspected it might be old, but no one had any idea quite how ancient. The eventual removal was dramatically filmed by Austrian television: the body was gouged from its icy grave with an ice pick and ski poles, wrapped in plastic and flown by helicopter to the nearest village, then driven to Innsbruck (subsequent investigation showed that the body's location was just on the Italian side of the frontier).

For 24 hours the body lay in the mortuary at Innsbruck University's Institute of Forensic Medicine, where it was photographed — and fingered — by members of the local press. The day after the body arrived in Innsbruck, Konrad Spindler, of the university's Institute of Archaeology, identified the accompanying metal axe as bronze, and the body was therefore ascribed to the early second millennium BC. Within hours, fungus was noticed on the corpse; it was handed over to the anatomy department whose staff, after seeking advice (though not, apparently, from palaeopathologists), wrapped the body in a sheet daubed with phenol and put it in a freezer at -6°C , where it still remains. The artefacts were taken to the Roman-Germanic Museum in Mainz.

Radiocarbon dating of the man's skin tissue and bone at Oxford and Zurich produced uncalibrated figures of about 4,525 and 4,575 years before present. When calibrated, these results place him between 5,100 and 5,300 years ago, the Late Neolithic period. Initial assessments of his morphological characteristics also placed him in the Late Neolithic and Bronze Age populations of south central Europe^{1,2}. The man was between

25 and 40 years of age (according to the degree of suture closure in the skull and the wear on the front teeth), between 1.56 and 1.6 m in height, and had a cranial capacity of 1,500–1,560 cm^3 .

Because the body's exact original position is unknown, it is difficult to establish the cause of death. It is thought that he was exhausted and froze to death; and he was clearly mummified (dehydrated) before becoming enclosed in ice, because corpses salvaged from glaciers normally have their body tissues transformed to adipocere (white grave wax). There are slight distortions to the lips and nose, attributed to "slight ice pressure due to glacier flow in the valley"¹.

But there is a considerable list of problems and uncertainties with this picture, as discussed both in a popular science article⁵ and in a series of television documentaries by Jean-Michel Bader, a French journalist, and Michael Heim of Bavarian State Television. Some of these doubts stem directly from the confusion, delays and lack of care that marred the early days of the discovery⁶: some of the people who visited the site between the body's discovery on 19 September and its removal 4 days later seriously damaged the corpse, even digging it out of the ice almost entirely and 'replacing' it.

There is a different set of problems concerning the dating. None of the varied range of artefacts found with the corpse has yet been dated. They should

have been dated at the same time as the body, but there was no coordination between the institutes of Anatomy (corpse) and Archaeology (artefacts). A similar absence of coordination explains why grass from his boots was dated separately at laboratories in Uppsala and in Gif-sur-Yvette, initially without the knowledge of those dating the corpse. The first results, reported to the media in December 1991, were $4,250 \pm 70$ (Uppsala) and $4,230 \pm 90$ (Gif) years BP (calibrated to 4,616 – 4,866 years BP). These were modified in January 1992 to $4,550 \pm 60$ (Gif) and $4,450 \pm 75$ (Uppsala), tallying more closely with the dating of the corpse. Again the dates were reported to the media, but have not been published either as part of the official reports or in the formal literature. Comprehensive dating of the artefacts would greatly help to clarify the situation, yet even now there are no plans to do this. The metal axe blade, first thought to be of bronze, was shown on analysis to be copper, which fits better with the corpse's date. However, as travellers in high mountains know well, weight needs to be kept to a strict minimum, which makes it particularly puzzling that the man's bow and 12 of his 14 arrows were unfinished, whereas his other weapons (dagger and axe) were worn by prolonged use. Another puzzle is that the corpse was naked⁶; if the body had been encased in ice for thousands of years until a short time before its discov-

IMAGE
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First photograph of the Iceman, taken as he was discovered by Helmut and Erika Simon.

H. and E. Simon

ery, why were its clothes lying some distance away? This may be related to cause of death, for people suffering from hypothermia remove their clothing⁷.

A more crucial enigma is the corpse's state of preservation. Even the slowest glaciers are renewed every 500 to 600 years, the time it takes them to flow from their summit to the base of the tongue, and they disgorge everything they contain. Any corpse found within a glacier is usually crushed or torn to bits: for example, the body of what is thought to be a Swiss mercenary from the sixteenth century was fragmented over a 100 m² area when found in the 1980s (ref. 8). The average static pressure of a glacier is 14 to 20 tons m⁻², and its mass is filled with convection currents, faults, subduction zones and rocks. How, then, could Similaun man have remained intact *in situ* for more than 5,000 years? His preservation is little short of miraculous, even allowing for the alleged protection from the depression in which he was found. The destruction of the body's original casing of ice has prevented any possibility of dating gas trapped in its air bubbles or studying the pollen within it, and hence proving when it became ice-bound.

The body's condition is equally astounding, as was noticed from the start by the late Rainer Henn, the original pathologist. The official hypothesis is that the corpse was mummified by a warm wind, an autumn *foehn*, which dehydrated it before it became trapped in ice: but a wind of that heat at this altitude seems unlikely, especially as the *foehn* blows at much lower altitudes than the height at which the body was found. The corpse still has all its soft tissue, including the eyes; it was not attacked by scavenging animals. The official explanation for this protection is that the body was quickly covered by a thin layer of snow — but it is hard to reconcile this suggestion with a dehydrating wind.

Furthermore, the body is completely impermeable, whereas a tanned leather bag and quiver found with it are porous and sodden. Henn was astonished that the body was apparently insensitive to temperature changes between its discovery and removal: usually wrinkling of the skin and alterations of the mucous membranes would occur. Could the man have been deliberately mummified before burial? We may never know, since the sheet daubed with phenol, in which he was wrapped for a time, may pose problems for chemical analysis.

The final puzzle is that the corpse's genitalia are missing. The study team assumes that they were probably broken off during excavation; but the possibility remains that the body was castrated before death. In addition, rumours are rife — and have been reported in several

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Beset by delays and lack of care.

homosexual publications in mainland Europe and in Britain — that sperm has been found in the corpse's anal canal and that the study team is hushing up this point out of embarrassment. In our opinion it is far more likely that the team thinks the rumour is too ridiculous to merit attention; but for posterity's sake it should make a clear statement.

Bader and Heim⁵ list several of these inconsistencies, but stop short of declaring the corpse to be a fake. They imply that they would not be surprised if some of those involved were engaged in a very elaborate hoax. It is hard to take the hoax hypothesis seriously, but the existence of these doubts and of lurid rumours is directly attributable to the appalling delays in mounting a proper, scientific investigation of the corpse.

One factor in the delay has been the dispute over the body between north Tyrol (Austria), south Tyrol (Italy) and Rome. It is regrettable that, in the absence of a pan-European authority, science has fallen prey to territorialism. Professor Werner Platzer, of Innsbruck's Institute of Anatomy, has said that "the research will be world wide": yet, despite the eventual inclusion of a few specialists from further afield, the scientific team which has been assembled is heavily dominated by Austrians and Germans, few of whom have any previous experience of bodies of great antiquity, and among whom there has been a lack of coordination. Bringing in more specialists from elsewhere could not only benefit the investigation but would help to pay for the research. It seems unrealistic for Innsbruck University to raise the money to pay for all the research, as Platzer has said he wishes to do, and this has further hampered progress.

Articles in the Austrian press have publicized half-truths and accusations; Similaun man passed directly from the glacier into what Bader and Heim have

called a media circus. Although the body was at first perceived as a potential source of considerable revenue, most opportunities have been squandered. The team of lawyers appointed to negotiate with the media has failed to raise significant funds, yet has turned down substantial offers of money from television companies and publishers. Scientific broadcasters have had almost no access to official images or data, or to laboratories carrying out research on the corpse. It has been reported in the Innsbruck press that the university's lawyers have charged it 4 million schillings (£228,000) for their services but, while denying the accuracy of the figure, both sides refuse to clarify the situation. Quoted in the Austrian magazine *News*, Dr Hans Moser, Rector of the university, said recently that "The Ice-man brings us no money, but he costs a fortune", and even, with a heartfelt sigh of despair, that "sometimes I think, let's get a shovel, and then we can bury him again!"

Eighteen months after the discovery, the amount of published information on the body and its associated finds is still pitifully small. Those in charge of the corpse still have not agreed on how to take samples of the internal organs, and further finds last August at the site have not yet been sent to Mainz to join the other artefacts. They remain in a freezer in Italy. Research opportunities have already been missed, and the delay in providing convincing answers to the many troubling aspects of the case has allowed bizarre rumours and whispers of fakery to circulate, bringing archaeology in general, and this find in particular, into disrepute. To salvage something from this sorry state of affairs, a serious analysis, calling on the expertise of the world's foremost specialists, has to be carried out very soon to obtain the maximum information from this unique and truly miraculous find, and hence lay to rest the nagging doubts that remain about the iceman's circumstances. □

Paul G. Bahn is a freelance writer on archaeology at 428 Anlaby Road, Hull HU3 6QP, UK. Katharine Everett is at BBC Television, and produced the 1992 *Horizon/Nova* documentary on the iceman. An updated version can be seen in the UK at 8 p.m. on BBC2 on 8 March.

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A disappointing decade of AIDS

Hopes that research would quickly yield a prophylaxis for AIDS, or possibly a cure, appear to be evaporating, together with the belief that infectious disease is historical only.

THE organizers of this year's international AIDS conference, arranged for June in Berlin, are fearful that potential participants from elsewhere will be deterred from taking part by news reports of attacks on foreign visitors to Germany. In a statement last week (see page 6), they sought to reassure visitors by referring to the brave stand on xenophobia taken by the federal German president, Dr Richard von Weizsäcker, among others. The hope seems to be that there will be 15,000 people in Berlin in June, as there have been at earlier annual conferences in this series (which alternates between a US venue and one elsewhere).

In reality, this year may be different for reasons other than participants' fear of being attacked. One obvious difficulty is the general impression that the annual AIDS conferences are no longer a preferred means of describing the results of new research, but instead are occasions of a more theatrical character. To the extent that they throw together researchers in the field and those who suffer from AIDS, or at least are infected by the human immunodeficiency virus (HIV), they are a vehicle by which the patients may express their frustration, even anger, that research has done so little for them.

This year, there may well be even more of that than previously. To put it mildly, AIDS research has lapsed into a kind of silence. That does not mean that nothing is being done, of course. On the contrary, there is a substantial army of people at work on the characterization of the strains of HIV responsible for individual infections, while the detailed parameters of different epidemiological models are being steadily if slowly refined. But it seems a long time since Mrs Margaret Heckler, then the Secretary of Health and Human Services in the US administration, was talking eagerly of a vaccine against HIV. In fact, that was nine years ago next June. It is not surprising that the field is rife with disappointment.

For what it is worth, this is far from being a unique occasion when the high promise of some field of research has turned out to be less attainable than serious people believed. In 1958, for example, *Nature* published a clutch of papers from the UK Atomic Energy Research Establishment at Harwell purporting to have demonstrated that thermonuclear fusion (not the cold kind) could be brought about in a laboratory apparatus (181, 217–233; 1958). Only shrewd Lyman Spitzer of Princeton expressed doubts on

the subject (181, 221–222; 1958). Most of those involved were already sketching the designs of the working fusion reactors they saw being thrown up around the world. Now, nearly half a century later, a great deal has been learned about hot plasmas and their confinement, but nobody would now dare say whether thermonuclear reactors will ever be feasible in the sense of being economic.

AIDS seems now to be in the same case. The obvious prophylactic, a vaccine, must be intrinsically more difficult to develop than Heckler and her advisers could have imagined. How do you set about destroying all T cells harbouring the retrovirus HIV, perhaps as complementary DNA integrated within the genome, without causing an immune deficiency as damaging as that of AIDS itself? If, on the other hand, the pathogenesis of AIDS resembles that of an autoimmune disease, which is plausible enough, it is prudent to remember that there are no systemic prophylactics for other candidate autoimmune diseases.

Quite how a vaccine, if there were one, would be used is also perplexing. Would it be administered to all children before puberty, or to the groups known to be most at risk, and what would be made of it in the poor countries of Africa, where the incidence of HIV infection is alarming, but where health budgets are usually too small for even diagnostic tests for HIV infection to be affordable?

The search for drugs effective in the treatment of patients with AIDS has also been frustrating. Wellcome's AZT, like other inhibitors of DNA replication, is palliative only, and understandably has damaging side-effects. The search for drugs which, perhaps in conjunction with AZT, may offer better protection is promising but necessarily slow. It should be within the power of the pharmaceutical industry to improve on the methods of treating the secondary infections to which AIDS patients succumb, but the frequency of Kaposi's sarcoma in some kinds of AIDS patients is not, as yet, understood.

What, in these circumstances, should be our view of the place of AIDS in the modern world? And how should rich countries like ours respond? To say that the outcome of the research of the past ten years has been disappointing does not imply that research should be abandoned. To understand the pathogenesis of AIDS is an even more important goal now than when it was thought there might be a vaccine around the corner.

So too is a better understanding of the

way in which the existence of a substantial pool of people with serious immune suppression may contribute to the emergence of virulent forms of other infections, tuberculosis for example. Is there really a connection between AIDS and the emergence in New York of tuberculosis strains immune to BCG, for example? But, at least temporarily, the interests of people with AIDS suggests that there should be a more vigorous attempt to treat the secondary infections, symptoms though they are.

That amounts to acknowledging that AIDS will be among us for some time yet, and that we must learn to live with it as safely as we can. That means that the condom is no longer just a contraceptive, but a medicine. To recognize it as such requires a greater degree of openness about sexual practices than many people find palatable. But there is no choice.

There is also an urgent need to meet head-on the anger now common among the community of AIDS patients, especially in the United States. It is an almost palpable phenomenon. Many carriers of HIV believe they have been given a death sentence by their physicians and hold, however irrationally, that the establishment is bent on doing them down; intravenous drug users and male homosexuals, already persecuted as they see it, are naturally the most prone to share this socially corrosive resentment. Wild advocacy at the outset of the epidemic of quarantine for those infected did not help.

How else to blunt this anger except by making sure that AIDS patients are dealt with sympathetically and civilly, and that they are given the best care that can be afforded? That does not imply that AIDS patients are intrinsically more deserving of care than people with, say, some fatal form of cancer. It is merely that they are the unlucky victims of a common fallacy of just a decade ago, the belief that fatal infections had either been eliminated or would soon be eliminated.

Meanwhile, it is only seemly to record that little headway is likely to be made against the incidence of AIDS, in some of the poorer countries of Africa for example. If there were the funds for diagnostic tests, it might be more prudent to spend them in other ways, to combat intestinal diseases in infancy for example. But if things drag on as they are for another decade, that is what will make us feel badly.

John Maddox

Extracts from a talk at Green College, Oxford, 9 February 1993.

Amyloid precursor on the G_o

Michael R. Hanley and Dennis J. Selkoe

IN Alzheimer's disease, distinctive extracellular plaques which are principally composed of an aggregated peptide, β -amyloid, accumulate in parts of the brain involved in memory and cognition. Much attention has been paid to the possibility that the plaques may be an early and critical feature of the disease, and direct¹ or indirect² neurotoxic effects of β -amyloid-related peptides have indeed been reported. But little is known of the normal functions of the parent polypeptide, the β -amyloid precursor protein (APP), and whether perturbations of its functions contribute to the disease pathogenesis.

On page 75 of this issue³, Nishimoto *et al.* propose a novel possibility — that APP is a neuronal receptor regulating the heterotrimeric GTP-binding protein, G_o. Moreover, they suggest that the proteolytic release of the β -amyloid fragment may leave behind a tonically active carboxy-terminal portion of APP which could injure neurons through constitutive stimulation of a cell signalling pathway involving G_o. In this picture, plaque formation from β -amyloid would be only a symptom, not a cause, of the neuronal injury occurring in Alzheimer's disease. This is a provocative proposal which raises a number of questions.

From their earlier work describing an apparent interaction between the receptor for IGF (insulin-like growth factor) II and another trimeric G protein, G_i (ref. 4), Nishimoto and colleagues predicted that G proteins interact with the cytoplasmic domain of APP. They now describe³ a potential 'consensus' amino-acid sequence for G-protein recognition, and they identify a stretch in APP₆₉₅ from histidine 657 to lysine 676 ('peptide 20') which conforms to this pattern. Using synthetic peptide 20, the authors report that this sequence caused a transient (maximal at 5 minutes) increase in [³⁵S]-GTP- γ -S binding to purified G_o but not to other purified trimeric or monomeric G proteins. This rapid enhancement of GTP- γ -S binding was augmented by adding part of the hydrophobic transmembrane sequence of APP to peptide 20 and was abolished by inactivating ADP ribosylation of G_o using pertussis toxin.

These results suggested that a sequence present in the cytoplasmic domain of APP might activate a trimeric G protein. If so, a physical complex between APP and G_o is required, and two lines of evidence support that notion. First, using solubilized brain membranes, immunoreactive α - and β -subunits of G_o were co-precipitated with

APP by an anti-APP monoclonal antibody. Second, using baculovirus constructions to express wild-type and deletion-mutant forms of APP₆₉₅ in cells, immunoprecipitated APP from detergent extracts of these cells was shown to bind purified G_o. Two APP forms retaining the peptide 20 sequence were able to bind G_o, whereas one lacking this region was not.

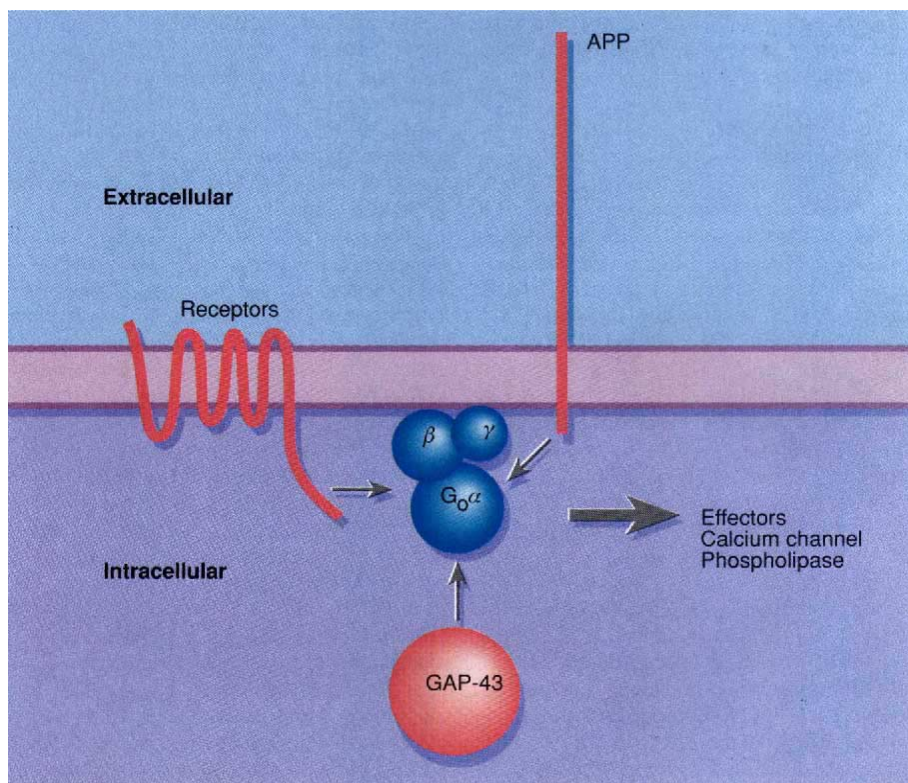
This interaction is clear-cut *in vitro*. But can it be demonstrated in living cells and is it physiologically relevant? These questions take on particular force because of another surprising interaction of G_o with a neuronal protein — the intracellular growth-cone protein, GAP-43, which binds G_o and activates [³⁵S]-GTP- γ -S binding⁵. There are some interesting comparisons between these two findings^{3,5}. For example, APP activation of G_o/GTP- γ -S binding is rapid and cannot be detected after 5 minutes, whereas GAP-43 activation is apparently persistent (it was detected at 60 minutes). Moreover, although the findings of both groups suggest that the active domains of APP and GAP-43 resemble cytoplasmic regions of G-protein-coupled receptors (which are known to interact with

trimeric G proteins⁶), "GAP-43 and peptide 20 may recognize different sites on G_o"³.

So it seems that G_o may be unprecedented as a trimeric G protein in its sensitivity to novel forms of receptor and non-receptor input (see figure). Such complexity might be more credible if it fitted in with expectations based upon the possible functions of G_o. Pinpointing those functions has been elusive, but G_o has been proposed as an activator of voltage-sensitive calcium channels⁷ or phosphoinositide-specific phospholipase C (ref. 8). If these are indeed effectors, elevation of cellular calcium might well be a final event, an appealing idea from the perspective of neuronal growth-cone modulation⁹ and possible neurodegenerative mechanisms¹⁰.

But there are other ways of interpreting a physical interaction of G_o with APP and membrane-associated proteins such as GAP-43. Perhaps G_o is bound to APP and GAP-43 not as a transducer, but rather as an intermediate in coordinating membrane traffic¹¹, particularly endosome formation and targeting¹². From this perspective, G_o interaction might be a common and essential component in neuronal membrane turnover and remodelling.

Intriguingly, Bomsel and Mostov have noted that "G_o may be involved in a membrane traffic pathway in neurons



Proposed neuronal interactions between the heterotrimeric G-protein G_o and seven-transmembrane-segment receptors, the amyloid precursor protein (APP) and GAP-43. Possible effectors are indicated, and GAP-43 is depicted as acting on G_o in a cytosolic, not membrane-associated state.

that is analogous to transcytosis, such as post-endocytotic sorting of membrane proteins into synaptic vesicles or polarized axonal transport¹¹. It is conceivable that alteration of APP processing coupled with augmented β -amyloid deposition during the early stages of Alzheimer's disease (at least in those cases bearing an APP structural mutation) could perturb a cascade of normal sorting functions, leading to disturbance of neuronal membrane architecture and turnover. Under such conditions, it is not hard to imagine that proteolysis of APP and formation and deposition of the β -amyloid fragment might increase, eventually leading to irreversible neuronal damage. Rethinking the context in which G_o might function could also resolve some of the controversies surrounding interactions of trimeric G proteins with proteins other than seven-transmembrane-segment surface receptors (for example, whether IGF-II receptor can signal or is only a transporter).

To address some of these issues, it will be necessary to examine the coprecipitation of APP not only with G_o but with other G proteins, GAP-43 and additional neuronal polypeptides, especially those associated with growth-cone membranes. The precedent of tyrosine kinase membrane receptors argues that functional complexes of membrane proteins may be elaborate¹³, and that many components involved in membrane traffic or transduction may form a dynamic unit. Another issue will be whether G_o might be involved in different types of neuronal injury or degeneration. If so, one crucial experiment will be to examine whether pertussis toxin is neuroprotective. In this respect, collapse of growth cones *in vitro* has been reported to require trimeric G proteins inactivated by treatment with pertussis toxin¹⁴.

The finding of an interaction *in vitro* between APP and a G protein opens up fresh prospects for research. But even if this process operates in intact cells, it

may relate more to the normal function and processing of APP than to its involvement in Alzheimer's disease. Several non-neural amyloid diseases involving other amyloidogenic proteins are characterized by the progressive deposition of a mutant and/or cleaved form of a normal polypeptide as amyloid fibrils, without necessarily perturbing the normal function of the parent protein. It remains to be seen whether increased formation and accumulation of the β -amyloid fragment, which is itself a nor-

mal product of cellular metabolism¹⁵⁻¹⁷, is accompanied by a phenotypically important change in the function of APP. □

Michael R. Hanley is in the Department of Biological Chemistry, School of Medicine, University of California Davis, Davis, California 95616, USA. Dennis J. Selkoe is at the Center for Neurologic Diseases, Brigham and Women's Hospital, 221 Longwood Avenue, Boston, Massachusetts 02115, USA.

ARCHAEOLOGY

No Eden in the New World

Karl W. Butzer

WHAT was the state of the environment when the Spaniards arrived in the New World in the sixteenth century? And what effect did they have upon that environment? Three assumptions have insinuated themselves into the public

between autochthonous, biogenic lake sediments and the organic matter, soluble material or unweathered minerals derived from the leaching and erosion of soils in the catchment area and deposited within the lake. These core profiles are

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

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Cortés meets Montezuma in Mexico (circa 1519) — were the imported Spanish farming methods any more destructive than those of the native Americans?

consciousness — that prehispanic agriculture was environmentally benign; that Spanish colonial forms of land-use, especially stock-raising, were highly destructive; and that, more generally, traditional farming systems are inherently conservationist and point to ways in which to reduce environmental degradation today.

On page 48 of this issue¹, O'Hara and colleagues provide convincing evidence for one centre of Mesoamerican civilization, around Lake Pátzcuaro in Mexico, that runs directly counter to these assumptions. They provide data from 21 cores that allow distinction to be made

dated and their microstratigraphy cross-correlated by 18 carbon-14 dates, primarily on charcoal and ostracodes; of these, 12 were measured by accelerator mass spectrometry to provide a remarkably consistent age framework of 4,000 years for the six lithological units identified, and one that is consistent with assumed dates for the regional archaeological sequence. This is a compellingly broad and accurate analytical database, far finer-grained than traditional pollen profiles. Moreover, unlike the more common, isolated limnological probes, it offers a three-dimensional picture of a catchment area and its

changing soil landscape over time.

The principal results are these. That there was an initial period of minor soil disturbance in the basin between about 1900 and 1250 BC (calibrated), coincident with the appearance of maize pollen. That a second, more intense episode of erosion occurred from about 600 BC to AD 650, linked to late Preclassic/early Classic occupation and concentrated in the northern part of the basin. And that a third, particularly destructive interval of soil erosion took place after AD 1200; this was initially centred on the Postclassic Tarascan (Purépecha) centre of Tzintzuntzan, and was followed by a slight decline in topsoil removal and a modest increase of mineral soil erosion after about 1500, during the Spanish and Mexican periods. Rates of soil-derived

sedimentation averaged 10,300 tons a year before about AD 650, 29,000 tons a year after about AD 1200, and 4,500 tons a year between these dates.

These figures show vividly that prehispanic indigenous land-use was not conservationist in practice. The curves suggest that an immediate response to reoccupation of the basin was an initial flush of topsoil, followed by a rapid, steady increase in mobilization of mineral sediment. Preclassic/Classic doubling of sedimentation rates implies that disturbance of the basin was then relatively modest, but Postclassic settlement provoked a sixfold increase and probably was environmentally destructive. The full scope of disturbance is barely hinted at in the coarse-grained pollen record from the basin², which does not support the impli-

cit notion of a deforested catchment. 'Shifting' hillslope farming, with fairly substantial fallow periods, today favours an irregular cover of secondary woodland in those humid parts of Mexico with indigenous-style farming, more consonant with the persistent oak and other deciduous, arboreal pollen represented in the palynological record.

A small Spanish town was founded at Pátzcuaro in 1537, but Spanish agricultural settlement was concentrated beyond the catchment, in Morelia; local Spanish land-use was probably limited to initial, mobile cattle-raising, soon followed by winter pasturing of sheep from drier parts of the plateau. Traditional, indigenous land-use persisted around the lake, despite a steadily declining Tarascan population; but the new bishop intro-

OBITUARY

Robert W. Holley (1922–1993)

In a remarkable decade from the mid-1950s to the mid-1960s, a series of discoveries established the foundation of our understanding of how genetic information is expressed in living systems. Robert W. Holley, one of the major figures of this period, died of lung cancer on 11 February at Los Gatos, California, north of the Salk Institute where he had been a resident Fellow for the past 25 years. Holley's principal contribution was in isolating and determining the nucleotide sequence of the first transfer RNA, so providing enormous insight into one of the fundamental events in protein synthesis.

His work started in 1956, on a sabbatical that he took at the California Institute of Technology from Cornell University, when he carried out experiments designed to detect the acceptor of activated amino acids. It was in the mid-1950s that Francis Crick proposed the 'adaptor hypothesis', that a small nucleic acid molecule might act as an adaptor for aligning amino acids along a ribonucleic acid chain during protein synthesis. During this period Mahlon Hoagland, Paul Zamecnik and others showed that radioactive amino acids became bound to a low-molecular-weight RNA, later called transfer RNA. It quickly became apparent that there were at least 20 classes of transfer RNA molecule, each capable of accepting an individual amino acid. To understand the process fully, it became necessary to isolate an individual species and then to determine its nucleotide sequence.

Holley's background made him well-suited to tackle this task. He had received a PhD in organic chemistry at Cornell in 1947, following his undergraduate training at the University of Illinois. He taught

organic chemistry and also worked in a laboratory of the US Department of Agriculture at Cornell. He selected the method of counter-current distribution, and developed it to the point where it



Robert Holley in 1968, the year he gained the Nobel prize.

could separate individual transfer RNA molecules which were assayed by their ability to accept a specific amino acid. Starting in 1958, it took three years of hard labour to isolate a purified species of the alanine acceptor transfer RNA from yeast. He began with 140 kg of commercial yeast and extracted 200 gm of transfer RNA which yielded 1 gm of the purified alanine species.

During the next four years, Holley developed methods that made it possible to determine the nucleotide sequence of this purified species. He introduced many innovations in that work, and the sequence was determined by using two sequence-specific ribonucleases which broke the molecule into smaller fragments that could be sequenced. Partial digests allowed him to obtain larger fragments which ultimately led to the sequence of the entire molecule. In the age of the Human Genome Project, in which we talk about kilobases of nucleotide sequence, it is important to realize

that Holley's effort represented the beginning of this process, albeit in the much more difficult world of RNA sequencing.

He was very interested in the secondary and tertiary structure of the alanine transfer RNA. Out of his work came the well-known clover-leaf secondary structure with the anticodon bases in one loop. Virtually all transfer RNAs sequenced subsequently have this clover-leaf arrangement; its full significance was realized in 1973, in X-ray diffraction studies that revealed the backbone folding of yeast phenylalanine transfer RNA, which produces an L-shaped structure with the anti-codon at one end and the amino-acid acceptor at the other end, more than 70 ångströms away.

Holley's elucidation of the nucleotide sequence of the transfer RNA molecule occurred in the same period during which the genetic code was being deciphered, and he shared the 1968 Nobel prize with Marshall Nirenberg and H. Gobind Khorana, who played major roles in deciphering the code. He moved to the Salk Institute in 1968, where he worked productively on factors that stimulate growth in mammalian cells.

Robert Holley was a modest man with a quiet and warm personality, an avid interest in science and a keen appreciation of the thrill of scientific discovery. As a hobby, he worked as a sculptor, and those who have seen his elegant bronze statues realize that he could have had another calling, had he elected not to go into science.

Alexander Rich

Alexander Rich is in the Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA.

ECOLOGY

Succeeding in the sand dunes

M. J. Crawley

duced Spanish technology here early on, probably including ploughs^{3,4}. Most notably, the limnological record does not support the idea of an increase in environmental degradation, even after the 1700s when both Hispanic and indigenous populations expanded rapidly. This evidence finds further support in the parallel pollen record from the adjacent catchment of former Lake Zacapu, once the site of a livestock hacienda⁵.

O'Hara *et al.* have provided a landmark study which shatters the myth of precolumbian America as an Eden in which people were "transparent in the landscape"⁶. Native Americans were populous and technologically skilled. The sustenance of those populations required agriculture and a vigorous use of resources that could be as damaging as any pre-industrial land-use in the Old World, as the record from Lake Pátzcuaro now reveals. Spanish forms of land-use were evidently no more harmful to the environment.

The implications of this conclusion are considerable, for there is a claim, increasingly heard, that indigenous agriculture was operated as a sustainable system in harmony with nature, suggesting an ideal norm for the future⁷. That is an oversimplistic view which ignores the points that the introduction of European livestock has greatly reduced the risk of starvation among rural peoples, has improved nutritional balance, and has provided ready fertilizer for small farmers in marginal agricultural areas⁸. Small-scale, traditional agriculture can, ideally, be adapted to local conditions and may deliver sustainable yields with minimal capital investment⁹; it is certainly preferable to 'technodevelopment'. But it would be inadvisable to consider it a total panacea, rather than as a source of information to be drawn upon in selecting more beneficial agricultural practices and strains of crops¹⁰. □

Karl W. Butzer is in the Department of Geography, and the Mexican Center, University of Texas, Austin, Texas 78712, USA.

ON page 53 of this issue¹, Van der Putten and colleagues describe a neat piece of work on the succession of plant species in sand dunes. They show that there is asymmetry in the effect of soil pathogens on plants earlier and later in the succession, and that this effect may in part drive the process of the replacement of one species by another. The

exhibiting deterministic events akin to birth, growth, maturity, senescence and death, and as a process in which facilitation of one species by its predecessor was a vital component. At the other extreme were the disciples of Gleason's³ individualistic concept of vegetation, who saw plant communities as mere accidents, as random assemblages from



W. H. Van der Putten

First colonist — a healthy stand of marram grass on virgin sand dune.

idea is at once enticing and contentious.

Succession is one of the few topics in ecology where the practitioners are disposed to make confident predictions. Species replace one another in a characteristic sequence (shade-tolerators replacing light-demanders, for instance), as each species progressively alters the environment so that conditions become conducive to the recruitment of their successor and hostile to self-replacement. Left alone for long enough, the ecosystem is supposed to progress serenely towards the climax vegetation appropriate to the local conditions of soil and climate. The very word 'succession' conjures up an almost regal orderliness in guiding a sequence of predestined dominants.

Where there has been controversy about plant succession²⁻⁵, it has been to do with the mechanisms responsible for the demise of the current dominant, and the extent to which species that occur early in succession facilitate the establishment of the later arrivals. At one extreme, Clements² saw successions as analogous to individual organisms,

the pool of species capable of existing under the prevailing conditions whose structure (such as it was) reflected little more than the vagaries of plant dispersal. In such communities, the principal mechanism affecting successional change was inhibition; once a species became established it simply stayed put, perhaps for a very long time. It monopolized the space and precluded the establishment of other, later successional species. Possession was nine-tenths of the law.

If inhibition is the main process determining the rate and direction of successional change⁶, what are the factors reducing the competitive ability of a dominant to the point that it becomes susceptible to invasion by its successor? Van der Putten *et al.*¹ provide an innovative experimental test of the hypothesis that species-specific soil pathogens can provide just such a mechanism. The authors chose to work on sand dunes, where the simplicity of the foredune succession allows one to assume that contemporary zonation in space reflects the succession of dominant species through time. The dune is built around

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the growth of marram grass *Ammophila arenaria*, which is followed in sequence by fescue *Festuca rubra* and sand sedge *Carex arenaria*, with sea couch *Elymus athericus* at the landward edge of the dune. But what causes the marram grass to become prone to invasion by the fescue, and the fescue by the sand sedge? Traditional explanations have included resource depletion, alteration of the physical environment, accumulation of metabolic toxins and the action of species-specific herbivores⁷. Van der Putten *et al.* now suggest that the breakdown of inhibition may be caused by soil-borne plant diseases.

The idea that soil-borne pathogens play a role in plant succession is not new. This is what Clements² was writing in 1928: "Parasitic fungi... restrict or prevent [establishment] either by the destruction of invaders or by placing them at a disadvantage with respect to the occupants" (page 78). "The most usual effect [of pathogens] is a decrease in number or dominance by which the species assumes a less important role. In the majority of cases no direct influence is discoverable, the effect being merged in the general outcome of competition" (page 91). What is exciting about the present study is the elegant test of the issues involved.

Van der Putten and colleagues carried out an ingenious series of reciprocal transplant experiments using sterilized and unsterilized dune sand. Two-week-old seedlings, raised from local seed, were planted into replicated series of pots containing sand collected from beneath each of the main dominant plant species; the sand in half of the pots was sterilized by gamma irradiation. They discovered that biomass production of each plant species was reduced by the soil-borne diseases of its successors but not by the diseases of its predecessors. This implies that soil-borne diseases affect both the rate and the direction of succession of foredune plant species.

If species-specific plant pathogens were the only mechanisms operating to break the inhibition imposed by dominant species, then the possibility of cyclical successions would arise. Suppose that species A is replaced by B, and B by C, but that by the time that C has attained dominance, the pathogens of A have died out, so that C can be invaded and replaced by A. The fact that we do not find marram grass invading lawns of sand sedge demonstrates that plant pathogens are only part of the story in sand-dune succession. The competitive ability of marram derives largely from its ability to withstand burial by sand, and in more stable soil conditions this competitive edge is lost so that marram could not re-invade, even if sufficient time had passed for its pathogens to have dis-

appeared. There is also an important element of facilitation in the dune succession because, in the absence of soil stabilization by the marram grass, the community could not be invaded by the other plant species. Thus, if the pathogens of marram grass were too effective, there wouldn't be any sand dunes for the other species to inhabit.

It remains to be seen how often species-specific soil pathogens are responsible for breaking the competitive stranglehold of early successional dominants. Long-term field tests involving pathogen exclusion using fungicides would be illuminating, although it is not clear how the effects of excluding pathogens and mutualist mycorrhizal species could be teased apart. Pathogens are most likely to be important in successions driven by inhibition rather than by facilitation, and in systems exhibiting strong dominance rather than in species-rich seral stages.

More generally, the hope is that rather than simply adding yet another element

to the long and growing list of complex and interacting factors, this study may help to reinforce the message that competitive ability is not a species-specific trait; it depends on the other species and on the circumstances (both biotic and abiotic) under which the struggle for existence is taking place. There is always more than one thing happening in succession, yet we may find that the variety of community dynamics reflects correlations among a small subset of ubiquitous mechanisms. □

M. J. Crawley is in the Department of Biology, Imperial College at Silwood Park, Ascot, Berkshire SL5 7PY, UK.

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ORGANIC CHEMISTRY

Polymer science branches out

Philip Hodge

AFTER years of seeking, for the most part, to synthesize polymers in the form of simple long linear chains, polymer scientists are turning their attention to more elaborate topologies. Dendritic polymers, or 'dendrimers'¹, are one novel type that has attracted a great deal of attention in recent years. Formally speaking, dendrimers are the result of several chains growing from a core molecule. As the chains become longer and further removed from the core they branch regularly and frequently so that the ever-increasing volume that is available is filled by an ever-increasing number of growing chains. Ideally all the chains grow equally and the final product is a close-packed molecular ball with many surface end groups. Hawker and Fréchet² at Cornell University have developed a new approach to dendrimer synthesis which not only makes the dendrimers more accessible and more

defect free, but which also allows blocks of copolymers to be assembled in one dendrimer.

One reason for the interest in dendrimers is that their architecture is so different from that of the traditional linear step-growth polymer. An ideal molecule of the latter type is one long chain with only two end groups. If the polymer has a substantial molecular weight, the end groups and their functionality, if any, are usually of little importance. The linear chains will usually exist in solution as flexible loose random coils. In contrast, the dendrimer has many short chains, a very high degree of branching and many chain ends (typically hundreds). In solution, it forms a tightly packed ball. The end groups at its surface are the main feature a dendrimer presents to other molecules.

Dendritic polymers were first described by Tomalia³ and co-workers in

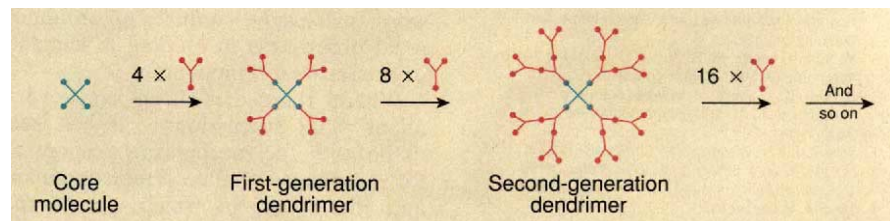


FIG. 1 The 'divergent' synthetic approach — building up a dendrimer from the inside out to create a 'starburst'. The blobs indicate the site of activity (actual or potential), or the site of a linking reaction.

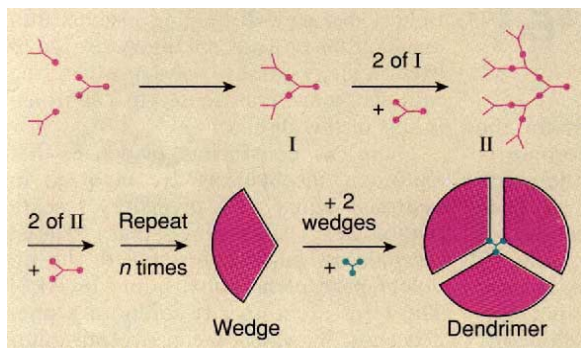


FIG. 2 'Convergent' synthetic approach — assembling a dendrimer in segments. The monomer units that will make up the outer layer have only one site where links can be formed, so the successive steps of the synthesis build up a segment from the outside in. Finally, the 'wedges' are linked at their apices to a core molecule.

1986. They used a divergent synthetic approach: starting at the core and working outwards. The basic idea, which has since been developed and widely exploited, is shown schematically in Fig. 1. It starts with a multifunctional core molecule: in this illustration the core has four functionalities. These are then reacted with four monomer molecules, each of which has two functionalities which will allow further monomer molecules to become attached, so giving the first-generation dendrimer. The process is then repeated for several generations.

In reality, the linking process is more complex than this and 'blocking groups' must be used to control which groups react at each stage. The cycle of reactions leading to each generation of dendrimers is completed as a series of practical steps before the next cycle of reactions is commenced. The total number of monomer molecules added soon becomes very large: after five generations the dendrimer contains more than a hundred monomer units, which would be a respectable number for many linear polymer chains. Using this divergent synthetic approach it is possible to prepare up to ten-generation dendrimers with molecular weights of up to 700,000 and with more than 3,000 end groups per molecule. But there are snags. For example, the conversion of one generation into another may not be complete, and it is difficult to detect whether this is so. If it is incomplete, separation of the resulting mixture of dendrimers is virtually impossible.

Instead, Hawker and Fréchet have synthesized dendrimers using a convergent approach. Here the synthesis starts at what will eventually become the periphery of the dendrimer and progresses inwards. Successive reaction cycles ('blocking groups' are also needed here) generate a 'wedge' with a reactive group at the apex (Fig. 2). The last step of the synthesis is to attach several wedges, by

means of their apical functionality, to a multifunctional core molecule.

This novel synthetic approach has several advantages, not the least being the ease of purification at each step. Because each step involves adding two large units to a smaller bifunctional unit, the desired product has a molecular weight very different from those of the units from which it is constructed. It is thus relatively easy to purify the desired product. The efficiency of the reactions is also easily monitored spectroscopically at each stage. These features help greatly to minimize the occurrence of 'defects' in the final structure.

An example of the power of the convergent approach is the synthesis

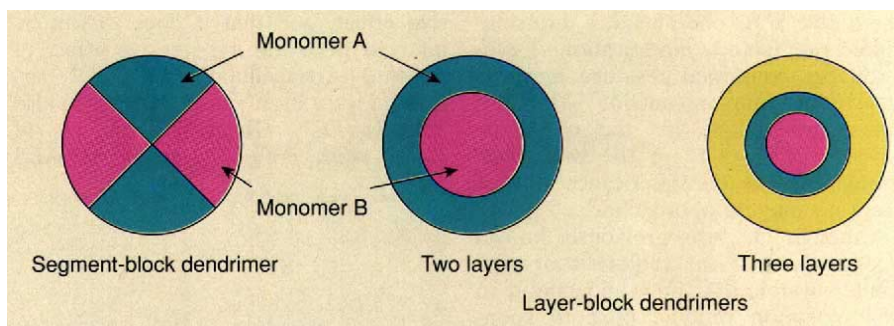


FIG. 3 Dendrimers synthesized by the convergent approach can be made up of blocks of different monomers.

of segment-block dendritic copolymers (Fig. 3). These are obtained simply by attaching different wedges to the same core molecule. Clearly the chemistry of the latter has to be such that the number of wedges added at each stage is controlled, but this is not difficult. Using this approach Hawker and Fréchet have synthesized a segment-block dendrimer with a molecular weight, M_n , of 5,364 which has one ether-linked segment and two ester-linked segments.

The copolymers can be made to lie in concentric spheres rather than adjoining segments. Hawker and Fréchet's example is the synthesis of a layer-block dendritic copolymer with a molecular weight, M_n , of 10,242. The inner two layers were ester-linked and the outer three ether-linked. In such layer-block dendrimers, the chain length of each block may be very small but the layer is a whole shell. In conventional linear polymers, the chain lengths of blocks are long but they have no 'width'.

Interest in dendritic polymers will continue because the degree of structural control available is much higher than

with linear polymers. Especially attractive features are monomer sequence control in copolymers and molecular weight control.

What possible applications are there for dendrimers? One possibility is a drug delivery system. This might be a layer-block dendrimer with surface groups that make the polymer water-compatible, ester linkages to make it biodegradable, and inner layers built of appropriate amounts of drug so that when the polymer degrades a uniform supply of the drug is delivered.

Segment-block dendrimers might find uses in enzyme reaction studies. Micelles, which are loose spherical arrangements of amphiphilic molecules with the lipophilic groups inside and the hydrophilic groups outside, have long been used in such studies. A molecule generally slips in between the lipophilic groups with the active part in the hydrophilic region. The hydrophilic head groups contain chemically reactive sites. In future, dendrimers might be prepared in

such a way that small molecules could creep into clefts between segments. The system could be more highly organized, have better recognition and be more permanent than micelles.

Novel materials might be obtained by close-packing dendrimers, then cross-linking them through surface functionality. And if the ins and outs of dendrimers begin to lose their novelty, other topologically fascinating polymers abound. Interest is now growing in other types such as rotaxanes (where a large cyclic compound is strung like a bead on a polymer chain), cyclic polymers, and catenanes (two cyclic polymers linked like a true chain). Polymer science, like the dendrimers themselves, is branching out in all directions. □

Philip Hodge is at the Department of Chemistry, University of Manchester, Oxford Road, Manchester M13 9PL, UK.

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Did radicals strike Lou Gehrig?

James O. McNamara and Irwin Fridovich

AMYOTROPHIC lateral sclerosis (ALS) is a late onset, inexorably progressive and ultimately fatal degeneration of motor neurons leading to progressive paralysis. It is also called Lou Gehrig's disease, after the baseball hero afflicted by it, and is now best known as the hellish condition which robbed Stephen Hawking of the control of his own body while leaving his intellect intact. On page 59 of this issue¹, Daniel R. Rosen *et al.* convincingly demonstrate that a familial variant of ALS is linked to defects in the SOD1 gene, which encodes the cytosolic superoxide dismutase (SOD). This enzyme, which contains both Cu(II) and Zn(II), catalyses the conversion of superoxide into hydrogen peroxide plus dioxygen:



The native SOD operates at a diffusion-limited rate, so any modification of evolutionarily conserved residues must be expected to impair function. The biological production, and the enzymatic scavenging, of $\text{O}_2^{\cdot-}$ are the twin gates through which the significance of this discovery may be approached.

Although $\text{O}_2^{\cdot-}$ was previously known as a product of the radiolysis of oxygenated water, it is now understood to be a common product of both spontaneous and enzyme-catalysed oxidations. It is chemically active both as a reductant and as an oxidant. It can, moreover, beget other more indiscriminately reactive entities. The SODs control this 'bull in a china shop'.

The clearest indication that SODs are essential comes from studies done with *Escherichia coli*. Although the wild-type *E. coli* grows in a glucose-plus-salts medium, either anaerobically or aerobically, a SOD-null strain is oxygen-sensitive and grows only anaerobically. It is, moreover, ultrasensitive towards compounds that increase intracellular production of $\text{O}_2^{\cdot-}$. Transformation of the SOD-null with a plasmid bearing a functional *sod* gene restores oxygen tolerance². Any functional *sod* gene will do, and such complementation of the SOD-null has been demonstrated using genes from eubacteria, plants or mammals.

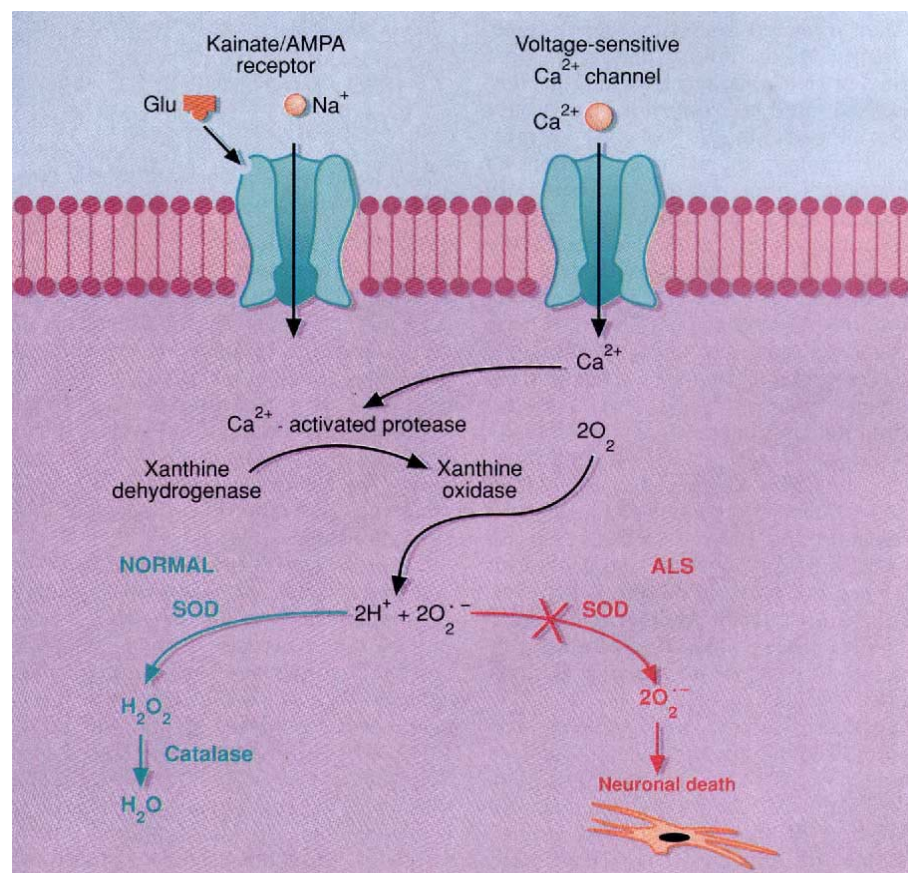
Humans contain three distinct SODs, each of which occurs in a different place — a homodimeric Cu,ZnSOD is found primarily in the cytosol, a homotetrameric MnSOD in the mitochondrial matrix, and a homotetrameric glycosylated Cu,ZnSOD in the extracellular spaces. The gene for the human cytosolic SOD

is on chromosome 21 and that for the mitochondrial SOD on chromosome 6; the chromosomal location for the extracellular SOD gene has not yet been reported. Given the short half-life of $\text{O}_2^{\cdot-}$ in the biological milieu, and its inability to cross lipid bilayers except through anion channels, it is not surprising that $\text{O}_2^{\cdot-}$ produced within any compartment must be dealt with by a co-localized SOD.

Superoxide is involved in numerous physiological and pathological processes. It is released in abundance during the respiratory burst of activated phagocytic leukocytes and plays a role in inflammation. Nitric oxide, the endothelium-derived relaxing factor, reacts very rapidly with $\text{O}_2^{\cdot-}$ which limits NO lifetime and decreases its net relaxing effect on the smooth muscle of blood vessels. Superoxide should thus exert a hypertensive effect, and that it does so can be inferred from the hypotensive effect of injected extracellular SOD³. Reperfusion injury of numerous tissues^{4,5}, also involves $\text{O}_2^{\cdot-}$. Given the findings of Rosen *et al.*, is familial autosomal ALS

now to be added to the list of free-radical diseases? Emerging insights into mechanisms of neuronal injury related to excitotoxicity point to ways in which free radicals may contribute to neuronal injury in this disease.

There is convincing evidence that excitotoxic mechanisms are involved in neuronal injury in a diversity of acute pathologies, suggesting that related mechanisms might contribute to the indolent pace of neuronal injury in ALS. The term excitotoxicity refers to a phenomenon in which the neurotransmitter glutamate and related compounds destroy neurons through an excitatory mechanism (glutamate is thought to be the principal excitatory transmitter in the central nervous system). In acute neuronal injury due to ischaemia or hypoglycaemia, abnormally high concentrations of glutamate accumulate in the extracellular space and excessive activation of neuronal glutamate receptors ensues, thereby literally exciting glutamate-receptive neurons to death. Epidemiological evidence hinted at a link between ALS and glutamate when ingestion of excess amounts of a glutamate analogue β -N-methylaminoalanine (BMAA) was implicated in a form of ALS and Parkinson's dementia in Guam⁶. The aetiological role of BMAA was strengthened



A hypothesis according to which physiological activation of kainate/AMPA receptors on motor neurons generates a low level of $\text{O}_2^{\cdot-}$, superoxide anions accumulate, and death of the motor neuron results.

when non-human primates were found to suffer damage to their motor neurons when fed large amounts of BMAA⁶. More direct evidence of an excitotoxic mechanism for chronic injury of motor neurons is the finding that prolonged incubation (several weeks) of spinal-cord explant cultures with a glutamate-uptake blocker selectively kills motor neurons⁷. Among the receptor subtypes through which synaptically released glutamate might kill motor neurons, the non-NMDA or kainate/AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate) subtype is the leading candidate. BMAA appears to exert its excitotoxic effects through non-NMDA receptors⁸. Moreover, antagonists of non-NMDA receptors can protect motor neurons from death produced by glutamate-uptake blockers in spinal-cord explant cultures⁷; by contrast, NMDA receptor antagonists are ineffective.

How might a decrease of SOD1 activity explain an excitotoxic mechanism of motor neuron death? Insight into the signalling pathways mediating kainate-induced neuronal death provides an attractive explanation of how a decrease of SOD1 activity might result in death of motor neurons (see figure). Kainate-induced neuronal death requires influx of calcium⁹. Interestingly, kainate-induced death of cerebellar neurons in primary culture can be abolished by inhibitors of xanthine oxidase, a cellular source of cytotoxic $O_2^{\cdot-}$ (ref. 10). By analogy with other systems, this implies that the kainate-induced influx of calcium activates a serine protease which in turn catalyses the transformation of xanthine dehydrogenase to xanthine oxidase. Importantly, neurons can also be protected from kainate-induced death by addition of SOD itself. Thus kainate-induced neuronal death may be produced by $O_2^{\cdot-}$ generated by xanthine oxidase.

If this is correct, it seems plausible that physiological activation of kainate/AMPA receptors on motor neurons over a lifetime, combined with a partial decrease of SOD1 activity, might lead to a subtle excess $O_2^{\cdot-}$ and to the gradual death of motor neurons becoming evident in middle age. This proposal differs

from the traditional excitotoxic hypothesis, in that synaptic activation of glutamate receptors itself is physiological, but protective mechanisms regulating some of the molecular consequences of physiological activation are defective. Key aspects of this hypothesis are immediately testable in the spinal-cord explant culture model. If analogous mechanisms are operative in ALS itself, several therapeutic options emerge, including antagonists of kainate/AMPA receptors, inhibitors of proteases that convert xanthine dehydrogenase to xanthine oxidase, and scavengers of $O_2^{\cdot-}$.

Although motor neurons are clearly

preferentially susceptible to chronic excitotoxic injury⁷, the explanation for this susceptibility remains unclear. The model proposed here points to several possibilities, including a high synaptic density of kainate/AMPA receptors, less-effective calcium buffering systems, and a limited repertoire of $O_2^{\cdot-}$ scavengers. $\square$

James O. McNamara is in the Durham Veterans Affairs Medical Center, and in the Departments of Neurology, Neurobiology and Pharmacology, and Irwin Fridovich is in the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710, USA.

PALAEOCEANOGRAPHY

Measures of productivity

Ed Boyle

ICE-core records^{1,2} show that the atmospheric concentration of carbon dioxide decreased during past glacial periods. But why? Plankton growth in the polar ocean is a prime suspect, as CO_2 is captured through photosynthesis and put out of circulation when the organisms sink to the ocean floor. For climate studies, a good measure of plankton productivity has been sorely needed. Two such tracers have recently emerged: nitrogen isotope ratios, whose use is described by Francois *et al.* in a recent issue of *Paleoceanography*³, and $^{231}Pa/^{230}Th$ ratios, described by Kumar and co-workers on page 45 of this issue⁴. The results cannot immediately settle the question of whether the polar carbon cycle controls the swings in CO_2 concentration⁵⁻⁷, but they indicate shifts in patterns of plankton productivity during glacial periods.

The problem in the past has not been lack of evidence but the difficulty of disentangling the results. Carbon isotope ratios and cadmium contents preserved in planktonic foraminifera shells are clues to the availability of nutrients, but are also affected by processes other than productivity. The rate at which biogenic silicate accumulates — a good indicator of productivity in the modern ocean — is obscured in ancient sediments by changes taking place after deposition. Not surprisingly, tests of the polar carbon-cycle hypothesis have been inconclusive.

The hypothesis may seem at first sight to require that, during glacial times, polar nutrient concentrations must decrease and biological productivity must increase. Neither of these changes are in fact essential. The cold deep ocean has a higher partial pressure of CO_2 than the atmosphere, and the polar ocean serves as a 'leak' in the 'biological pump',

keeping this carbon from reaching the atmosphere (because productivity is not high enough to remove all of the nutrients and excess carbon that upwell in these regions). If all other aspects of ocean circulation remain constant, then both an increase in polar productivity and a reduction in surface nutrient concentrations are indeed needed to lower the atmospheric CO_2 concentration. But if the rate of upwelling and mixing in polar regions decreases, or if the alkalinity, nutrient and carbon concentrations of the upwelling feedwaters change, then it is possible to change atmospheric CO_2 without changing polar production or surface nutrients⁸. It is the efficiency of nutrient utilization, not the supply itself, that is the essential control. An absence of surface chemistry changes would not disprove the polar carbon-cycle hypothesis, but would restrict the characteristics of the upwelling feed-water (concentrations and flux) allowable in such models.

The $^{15}N/^{14}N$ ratio of bulk sedimentary organic matter apparently depends on the efficiency with which surface nitrate is utilized. The nitrogen isotope system is more complicated than the carbon isotope system. Basically, phytoplankton preferentially take up ^{14}N relative to ^{15}N , so that where nutrient supply exceeds demand (as happens in many upwelling regions), the organic matter that sinks to the sediment has a lower $^{15}N/^{14}N$ ratio than the dissolved inorganic nitrate of the upwelling sea water. In regions where the nutrients provided to the surface ocean are used completely, $^{15}N/^{14}N$ is the same in falling organic matter and upwelling sea water. Further fractionation of $^{15}N/^{14}N$ occurs during early sedimentary changes, but bulk organic $^{15}N/^{14}N$ shows patterns comparable to surface nutrient distributions in

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the Southern Ocean and equatorial Pacific, with higher $^{15}\text{N}/^{14}\text{N}$ associated with lower surface nitrate.

In a transect of cores from 40° to 55° S in the Indian sector of the Southern Ocean, Francois *et al.*³ observed that the transition from high to low $^{15}\text{N}/^{14}\text{N}$ shifted closer to the South Pole during the last glacial maximum. Within the modern transition zone at a given site, $^{15}\text{N}/^{14}\text{N}$ was higher in glacial times, implying that nitrate utilization was higher there than today. But the range of values across the zone was similar to that of the present, implying that nitrate uptake at high latitudes remained as low as it is at present (at least at 55° S). Given micropalaeontological evidence that the Antarctic Circumpolar Front moved northwards during the most recent glacial maximum, it also seems that nutrient uptake was higher in the region between the modern and glacial positions of the circumpolar front. Overall, the nitrogen isotope data imply increased nitrate utilization in a band north of the present circumpolar front, but not south of this zone. This is the region of formation of Antarctic Intermediate Water, so this observation may account for reports of decreased nutrient concentrations in intermediate waters of the Northern Indian Ocean⁹.

As Francois *et al.* admit, there are uncertainties in this interpretation: later changes will have overprinted the primary particle flux signal; the oceanic nitrogen isotope system is complex; and the nitrogen isotope signature of oceanic nitrate may vary over time. We may see new interpretations of their data as these effects are investigated in more detail. But their approach should certainly help to clarify the nutrient status of upwelling waters.

An entirely different approach to the problem is applied by Kumar *et al.* in this issue. They make use of the difference in 'stickiness' of the radioisotopes ^{231}Pa and ^{230}Th , which are generated uniformly throughout the ocean from the decay of dissolved ^{235}U and ^{238}U . Because ^{230}Th is highly 'particle-reactive', ^{230}Th generated in the ocean water column tends to attach itself to locally produced sinking particles which fall nearly vertically to the sea floor below. ^{231}Pa , on the other hand, is less particle-reactive, so that it can travel substantial distances through the ocean before becoming attached to a particle and falling to the sea floor. As a result, ^{231}Pa tends to migrate towards regions of highest particle flux, and the $^{231}\text{Pa}/^{230}\text{Th}$ ratio of the material falling to the sea floor reflects the productivity of the near-surface waters above the site.

Unlike all other tracers of past particle flux, this one is nearly immune to later modification; by the time they reach the

sea floor, both elements are strongly attached to particles and do not move about in solution. Although the particles and their attached isotopes can be transported some distance from their original site of deposition, $^{231}\text{Pa}/^{230}\text{Th}$ is unaffected by this movement, so its value in the sediment reflects regionally averaged production. Both isotopes decrease through radioactive decay after deposition, but their initial ratio can be easily calculated for the past 200,000 years if the age of the sediment layer is known.

Kumar and co-workers traced the history of $^{231}\text{Pa}/^{230}\text{Th}$ in two cores covering the past 150,000 years in the Atlantic sector of the Antarctic seas. The core at 54° S showed decreased $^{231}\text{Pa}/^{230}\text{Th}$ (lower productivity) during glacial times, whereas the core at 48.7° S showed increased $^{231}\text{Pa}/^{230}\text{Th}$ (increased productivity). The geographical difference resembles that seen in silica sedimentation rates from cores in this region¹⁰.

These two new approaches have the benefit of being directly related to productivity. From their results, as well as from earlier data on opal accumulation, it would appear that in glacial times there was a zone of higher productivity north of the present position of the Antarctic Circumpolar Front, and either a slight reduction or little change in productivity southwards. All of these indicators are inconsistent with a general increase of productivity in high southern-latitude waters during glaciation. Like the previous tracers (carbon isotopes and cadmium) they do not encourage the idea that the polar carbon system controls atmospheric CO_2 , although (for the reasons noted above) they cannot rule it out. If the picture provided by these new tracers is confirmed, the details of how the control operates cannot be as simple as envisaged in the original models. □

Ed Boyle is in the Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.

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Blue electrons

DAEDALUS has been musing on the curious fact that electrons are blue. At least, a solution of sodium in liquid ammonia is blue; and its colour is attributed to dissolved electrons.

What happens when yellow sunlight shines into such a solution from one side? Each blue electron that absorbs a fast-moving yellow photon will recoil away from the light source, thus generating a tiny voltage. These collisional voltages will add in series all along the light path. Daedalus calculates that a metre of light path might generate up to 4,000 volts, easily extracted by wire-grid or transparent indium-oxide electrodes which would not obscure the light.

No electrochemical change should occur. Electrons would simply enter the ammonia solution at the anode, nearest the source of light, while an equivalent number would be 'plated out' onto the cathode on the shadow side. Liquid ammonia, of course, is rather awkward stuff. At ambient temperature its vapour pressure is about 10 atmospheres. But organic amine solvents like diaminoethane also form blue electron solutions, so Daedalus hopes that a more convenient and tractable electrolyte can be developed.

A liquid-phase photovoltaic cell should have many uses. Its voltage need not be limited by the extinction depth of its blue solution. A narrow cell, with light shining obliquely into it, could be arbitrarily long. Each diagonally entering ray of light would induce a voltage over its finite light path; the voltage components parallel to the cell axis would be summed over its entire length. So Daedalus hopes to develop a solar-powered overhead communication cable, a sort of electro-optic fibre. Direct sunlight would enter such a cable obliquely most of the time, though the voltage and even the polarity might change with the time of day. In cloudy Britain, the pipe would have to be fitted with closely spaced conical louvers so that even diffuse daylight would hit it obliquely. The cable could charge storage batteries to continue the service at night: Indeed, with some cunning secondary-cell electrochemistry built into it, it might even act as a storage battery itself.

The inherently vast voltage generated by such a cable would be dropped to safe levels by the power drawn from it. But an open-circuit or lightly-loaded cable, with its even, linear summation of potential difference, could generate unprecedented voltages for high-energy physics research. Daedalus is dreaming of a trans-Australian cable from Darwin to Adelaide, generating gigavolts in the outback noonday Sun.

David Jones

Running to catch the ball

SIR — When fielders run backwards or forwards to catch a ball they run at a speed which keeps $d^2(\tan\alpha)/dt^2$ zero, where α is the angle of elevation of gaze from catcher to ball. This ensures that they intercept the ball before it reaches the ground (provided they can run fast enough to keep $d^2(\tan\alpha)/dt^2$ zero) whatever the effect of aerodynamic drag on the ball's trajectory.

We video recorded a skilful fielder as he ran to catch cricket balls fired into the air from a bowling machine at 20–25 m s⁻¹ and an elevation of 45°. The balls came directly towards him so he had to decide whether to run back, forward or stay where he was, but not whether to move left or right. His velocity as he ran to catch the ball is plotted against time on the left of the figure. He is shown running various distances from 2.9 m backwards to 8.4 m forwards. The curves end when he catches the ball. It can be seen that he is always running (he has a non-zero velocity) when he catches the ball. He does not run to the point where it will fall, and then wait for it.

The right-hand side of the figure shows $d^2(\tan\alpha)/dt^2$ for each run. In each case the dashed line shows the value the fielder would have observed had he remained stationary and the solid line shows the value he actually observed as he ran. His strategy is clear. He waits for about half a second, then starts to run, accelerating until he reaches a velocity where $d^2(\tan\alpha)/dt^2 = 0$. Then he modulates his speed up to the point of catching, maintaining $d^2(\tan\alpha)/dt^2$ close to zero.

It can be seen why this algorithm is

successful by considering what happens when the fielder misses the ball. If it drops to the ground in front of him, $\alpha = 0^\circ$. If it goes over his head, $\alpha = 90^\circ$. So any strategy which keeps $0^\circ < \alpha < 90^\circ$ throughout the flight will result in the fielder reaching the ball before it hits the ground or goes over his head.

To see why keeping $d^2(\tan\alpha)/dt^2 = 0$ ensures that α lies between 0° and 90° throughout the flight, consider what happens if the fielder starts to run while the ball is going up. In this situation, α will be increasing and $d(\tan\alpha)/dt$ positive. If he runs so that $d^2(\tan\alpha)/dt^2 = 0$, $\tan\alpha$ must be positive and finite at the end of the flight. As $\tan 0^\circ$ is 0 and $\tan 90^\circ$ is infinite it follows that α will lie between 0° and 90° at the end of the flight. Thus the ball will be intercepted. This is true independent of the effect of drag on the ball's trajectory, so it is a strategy for the real world, and not just a geometric curiosity which applies to intercepting objects in parabolic flight^{1,2}. This strategy does not tell the fielder where or when the ball will land. It simply sets him on a course which will ensure interception.

Children probably discover this somewhat obscure strategy for keeping α between 0° and 90° by extrapolating from their experience of watching balls thrown towards them. If they remain stationary only balls which are going to land in their arms produce a value of $d^2(\tan\alpha)/dt^2$ close to zero throughout their flight¹. When the child starts to run to catch the ball a natural strategy would be to try keeping $d^2(\tan\alpha)/dt^2$ low, as that has been associated with successful

catching when stationary. This 'strategy' is obviously not available consciously. That its effectiveness is discovered demonstrates the power of the brain's unconscious problem-solving abilities.

Peter McLeod

Department of Experimental Psychology,
University of Oxford,
Oxford OX1 3UD, UK

Zoltan Dienes

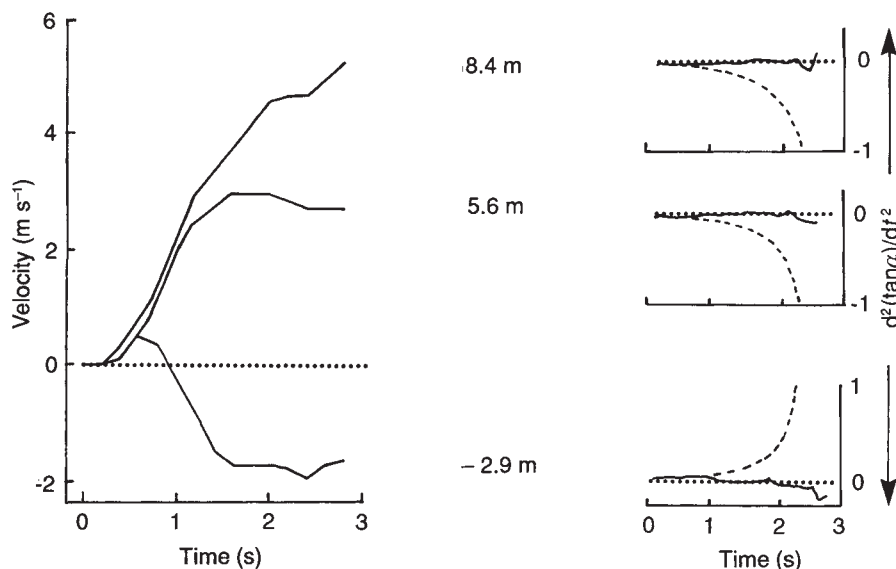
Department of Experimental Psychology,
University of Sussex,
Brighton BN1 9QG, UK

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Tetramer data reinterpreted

SIR — We recently described¹ a chemically crosslinked complex of relative molecular mass approximately 240,000 (240K) containing murine MHC class I heavy chains and β_2 -microglobulin. The complex was generated using two different crosslinking reagents, was immunoprecipitated with two different monoclonal antibodies specific for distinct class I alleles, and was observed in a range of cell lines. It was observed on cell surface iodination and also when crosslinker was added to cells in culture before quenching and lysis. On pulse-chase analysis, the concentration of the complex increased with time, suggesting temporal assembly, while a 140K chaperone-class I complex² disappeared in a reciprocal manner. We suggested that the complex, which seemed to contain only class I heavy chains (HC) and β_2 -microglobulin (β_2m), represented post-translationally assembled MHC class I tetramers. Since no other protein was seen by metabolic labelling or by iodination, we did not think it likely that any other protein was present in this complex.

At some intermediate concentration of crosslinker, intermediates of a final crosslinked product are expected to be generated and should be detectable on SDS-PAGE. If the 240K complex represents tetramers, the expected intermediates would be dimers and trimers. If it represents some other protein (P) cross-linked to class I, two bands (P-HC- β_2m and either P-HC or P- β_2m) would represent predicted crosslinking products. In our paper¹, we noted the absence of intermediate bands representing dimers and trimers. On a more systematic titration of crosslinker, we neither see the predicted dimers and trimers nor do we observe an intermediate product (P-HC or P- β_2m) that would have been predicted if the 240K complex represented some unlabelled protein crosslinked to



Left, the fielder's speed as he runs to catch balls landing at different distances in front of (8.4 or 5.6 m) or behind (2.9 m) him. Each curve is the mean of five successful catches. Right, the value of $d^2(\tan\alpha)/dt^2$ as he ran. The angle of gaze from fielder to ball (α) is estimated from film of the catcher's position and our model of the ball's trajectory.

class I (P-HC- β_2 m).

We cannot see the 240K complex with a third monoclonal antibody, B22.249 (specific for the $\alpha 1$ domain of class I heavy chains). We compared immunoprecipitation with covalently immobilized 28-14-8S antibodies (specific for the $\alpha 3$ domain of D^b) with our usual immunoprecipitation protocol with this monoclonal antibody added in solution, but the 240K species could not be seen when immobilized antibody was used, suggesting that this band might represent the covalent association of the monoclonal antibody with HC and β_2 m.

We cannot think of a simple explanation for the absence of intermediate species and the increase in the 240K species on pulse-chase analysis. It could be that a critical lysine residue reacts with the crosslinker only after HC and β_2 m are covalently crosslinked, and that this lysine-crosslinker hemimer is parti-

cularly refractory to quenching. Other recently obtained data are compatible with such a hypothesis.

We regret that this alternative interpretation to our data was not explored until very recently. This interpretation indicates that the 240K species is of no biological interest. Our new experiments do not affect the conclusions of studies using other physical techniques suggesting that MHC class I molecules form higher-order oligomers³.

Philippe Benaroch

Sudhir Krishna

Shiv Pillai

MGH Cancer Center,
Charlestown Navy Yard,
Boston, Massachusetts, 02129, USA

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Similarity of active-site structures

SIR — Crystal structures of hydrolases have shown similar conjunctions of active-site groups in enzymes hydrolysing different substrates. Catalytic structures resembling the serine protease catalytic triad in particular have now been found not only in serine proteinases, but in acetylcholine-esterase¹, lipases² and, in a modified form, in the structure of the RTE-1 class A β -lactamase from *Escherichia coli*³.

The 'generalized' serine proteinase mechanism involves two steps: formation of an acyl-enzyme intermediate with release of the first product; and the truly

hydrolytic step in which this covalent intermediate is cleaved. In the structure of the β -lactamase acyl-enzyme intermediate³, the residue glutamate 166 has been mutated to asparagine, preventing the second step, and trapping the acyl-enzyme intermediate. The role of Glu 166 has been controversial⁴, but appears from the structure described in ref. 3 to be involved in activating a bound water molecule to attack the ester bond of the acyl-enzyme intermediate.

We were struck by the remarkable structural similarity between the arrangement of the side chains of Glu

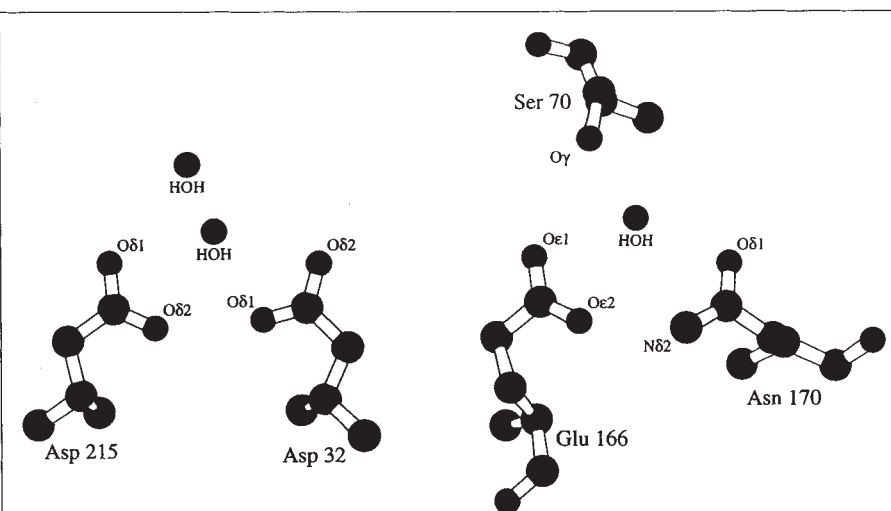
166 and Asp 170 and their bound water molecule in the β -lactamase structure³, and that of the side chains of the catalytic aspartates and their bound water molecule in the active sites of aspartic (and retroviral) proteinases⁵. As the coordinates for the *E. coli* structure are not yet available, we have taken the published coordinates of the similar β -lactamase from *Staphylococcus aureus*⁶ (P3BLM in the Brookhaven Protein Databank), and found that we can superimpose the side chains of Glu 166 and Asn 170, and WAT 81 (WAT 321 in the RTE-1 structure) from β -lactamase, with the corresponding atoms of Asp 215, Asp 32 and WAT 2 from endothiapepsin⁷ (P4APE in the Brookhaven Protein Databank) with an r.m.s. mismatch of only 0.33 Å. The difference between the two configurations results from the relative coplanarity of the carboxyl groups in the aspartic proteinase, compared with the slight buckling between the carboxyl and amide group planes in β -lactamase. The position of the water molecule relative to the side chains in both cases is extremely similar (see figure).

The functional role of these configurations is essentially the same in both classes of enzyme. In the aspartic proteinases, the two aspartic acid residues activate their bound water molecule, catalysing a nucleophilic attack on the peptide carbonyl of the substrate, whereas in the β -lactamase the activated water attacks the ester carbonyl of the acyl-enzyme. In both cases one carboxyl group acts as a general base, while the other group (protonated carboxyl in aspartic proteinases, amide in β -lactamase) provides a second hydrogen bond acceptor for the bound water and stabilizes the configuration via a hydrogen bond to the general base. In aspartic proteinases, the second carboxyl may also act as a general acid, facilitating nucleophilic attack by transfer of a proton to the peptide oxygen or nitrogen. The ester bond in the β -lactamase acyl-enzyme is much more susceptible to nucleophilic attack than a peptide bond and does not require this added 'push' to form the tetrahedral intermediate.

Laurence Pearl

Department of Biochemistry and
Molecular Biology,
University College London,
Gower St,
London WC1E 6BT, UK

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Analogous catalytic residues in endothiapepsin (left) and *S. aureus* β -lactamase (right). The corresponding nine atoms from each enzyme (C β , C γ and O δ /N δ atoms for Asp/Asn, C γ , C δ and O ϵ atoms for Glu, and the oxygen of the bound waters) can be superimposed with an r.m.s. deviation of 0.33 Å. When the structures are superimposed (matching Asp 215 with Glu 166 and Asp 32 with Asn 170), the two bound water molecules are <0.5 Å apart. A third hydrogen-bonding contact to the water is found in both structures, in a similar position; a second solvent molecule in endothiapepsin, and the O γ of Ser 70 (the catalytic serine residue) in β -lactamase. Figures produced using MOLSCRIPT⁸.

More on modified dynamics

SIR — Lindley's balanced comment¹ on the standing of 'modified dynamics' (MOND)² as an alternative to dark matter should bring the issue closer to the focus of attention. We would like to clarify a few points.

MOND is not just a modification of the inverse distance square law of gravity. There have been several attempts to explain flat rotation curves of spiral galaxies by modifying inverse square attraction beyond some critical length scale; the clear prediction of such a modification is that larger galaxies should possess larger discrepancies between the observable and newtonian dynamical mass, but this is not observed³. The fact is that discrepancies tend to appear at low accelerations, not at large distances, and this is the principal empirical motivation for MOND. Here, there is one new constant, a universal critical acceleration a_0 ($\approx 10^{-8}$ cm s⁻²) below which the law of attraction or of inertia takes on an unfamiliar but specific form. Because of this the theory, when viewed as a modification of gravity, not only involves a different distance dependence for attraction, but also a different dependence on the attracting mass.

We do not think the "abrupt transition from normal to acceleration dependent gravity" is an "untidy" aspect of the theory. The transition is not more abrupt than that from non-relativistic physics to relativistic physics, or from classical to quantum physics.

Lindley notes, on the positive side, the success of MOND in explaining flat rotation curves of galaxies without dark matter. But it is not just that MOND in some very general sense implies flat rotation curves; it predicts the exact form of the rotation curve for a specific galaxy from the observed distribution of stars and gas, in some cases more successfully than multi-parameter dark halo models⁴. In addition the theory predicts the observed form of the luminosity-rotation velocity relationship for spiral galaxies, the Tully-Fisher law; the luminosity-velocity dispersion relation for elliptical galaxies, the Faber-Jackson law; and the existence of a critical maximum surface brightness for spirals and ellipticals, the Freeman and Fish laws.

Beyond galaxies, it predicts the correct magnitude of the missing mass in clusters of galaxies and in the Virgo supercluster — all of this with one constant, a_0 .

Lindley echoes a recurring complaint that MOND is *ad hoc* and at present has no basis in deeper theory. We agree that, by and large, the rationale for the idea is phenomenological; however, the near equality of the fundamental acceleration and cH_0 points to a possibly profound connection with cosmology and renders the theory less *ad hoc*. There have been several attempts at deriving a relativistic extension of MOND⁵ and, although these theories contain physical anomalies, they do

demonstrate the possibility of reconciling MOND with standard Big Bang cosmology⁶. Of course MOND would gain considerable respectability by making some connection to more familiar physics, but its success on a phenomenological level cannot be ignored. It is because of this that the idea slowly begins to attract the serious consideration which it deserves.

M. Milgrom

*Department of Physics,
Weizmann Institute of Science,
76100 Rehovot, Israel*

R. H. Sanders

*Kapteyn Astronomical Institute,
9700 AV Groningen, The Netherlands*

Use of silk in ancient Egypt

SIR — On examining hair samples of mummies in the scanning electron microscope we found a piece of tissue between the curls which had the characteristic appearance of silk (see figure). To show that the specimen was silk, we performed infrared studies using multiple

Thebes, Deir el Medina, at the burial ground of the king's workmen. Based on anthropological data, the mummification method, the burial ground and amino-acid racemization, the mummy can be assigned to the twenty-first dynasty.

The silk industry had its origin in China and the material probably first reached the Mediterranean countries via Persia. Silk was not used in Egypt until later; the earliest example that can be traced is of Ptolemaic date from Mostagedda, a woollen tunic with decorative stripes with a weft of white silk. Lucan, writing in the middle of the first century, described Cleopatra with "her white breasts resplendent through the Sidonian fabric, which, wrought in close texture by the skill of the Seres, the needle of the workmen of the Nile has separated and has loosened the wrap by stretching out the web".

A portion of a coloured silk fabric was found at Qustul, south of Abu Simbel, the exact date of which is not certain, though it is probably not older than the fourth century AD. From the fourth century AD onwards silk became more common in Egypt. Our work suggests that silk was used in Egypt as long ago as 1,000 years BC, which would shed new light on ancient trading practices.

G. Lubec

J. Halaubek

C. Feldl

B. Lubec

E. Strouhal

*Department of Paediatrics,
University of Vienna,
A 1090 Vienna,
Austria*

Scanning electron micrograph of the textile specimen found in mummy's curl. Scale bar, 400 μ m. (Photo by R. Surenian.)

internal reflection, allowing nondestructive identification of the material¹. The spectra clearly identified silk.

We performed amino-acid analysis of the sample according to the method in ref. 2 and obtained the typical spectrum of hydrolysed silk, with high glycine, serine and alanine peaks as originally described by Shimura³. To exclude the possibility that the silk specimen could have been added later to the mummy's hair we performed amino-acid racemization studies on the mummy's hydrolysed hair samples and on the hydrolysed silk specimen: proline racemization was used as the marker amino acid. We used an HPLC method for the separation of L and D forms⁴.

The D/L racemization ratios from hair and silk were comparable, which excludes contamination of hair by the silk tissue in recent times. The mummy, a 30–50-year-old female, was found in

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Sporozoite invasion

SIR — Our understanding of the molecular basis of the invasion and subsequent development of malaria sporozoites in hepatocytes has been improved by recent reports that two sporozoite surface proteins, CS (ref. 1) and TRAP (ref. 2), both contain a sequence, region II, similar to the cell adhesion domain of thrombospondin³. Cox in News and Views⁴ reviewed work^{5,6} suggesting that recombinant CS protein containing region II binds to the sinusoidal face of hepatocytes and to the human hepatoma cell line HepG2, and that anti-region II antibodies significantly reduce sporozoite development in HepG2 cells. Cox suggested that these experiments show how malaria sporozoites invade hepatocytes directly from liver sinusoids, and that region II could be used as a target for immunization or chemotherapy.

However, region II probably binds to sulphated glycoproteins of the extracellular matrix. It is unlikely that this is sufficient in itself to mediate hepatocyte invasion specifically and subsequent development, which may, as suggested^{5,6}, require additional regions of CS or other sporozoite proteins. We have previously demonstrated that a peptide KLKQPGDGNPD (N1), spanning region I of CS protein, binds to HepG2 cells and specifically recognizes two HepG2 proteins of relative molecular mass 55,000 (55K) and 35K. Because anti-N1 antibodies inhibit sporozoite invasion of HepG2 cells⁷, region I is also likely to be involved in sporozoite invasion. When purified human hepatocyte membranes are crosslinked to sporozoites, two 55K and 20K proteins are identified which, when purified, each inhibit sporozoite invasion into human hepatocytes⁸. The sporozoite ligand(s) for these receptors remains to be identified.

A series of complex molecular interactions between sporozoite and hepatocyte molecules is therefore critical for sporozoite invasion and subsequent intrahepatic development. Region II-mediated adhesion is likely to be involved in the early stage of hepatocyte recognition. *In vitro* culture of malaria parasites in HepG2 cells alone is consistent with the suggestion that sporozoites invade hepatocytes in the space of Disse. However, the fact that hepatocytes rather than endothelial or Kupffer cells are labelled by region II does not indicate that sporozoites directly travel from the sinusoid to the space of Disse. Molecular interactions other than those in region II could be involved in endothelial or Kupffer cell recognition.

The relevance of these results to the development of anti-malarial vaccines based on CS protein remains an open question. CS vaccines containing non-repeat regions including region II are undergoing clinical trials. Although there are potential adverse pathological effects resulting from anti-region II antibodies that might recognize human thrombospondin, it is probably fortuitous that CS region II is naturally so poorly immunogenic. Other CS domains, or other sporozoite or liver stage proteins, must also be considered as candidate vaccines. In this context it is interesting to note that it is now becoming clear that entry of viruses into cells requires several ligands⁹. If there are parallels in malaria, then development of a vaccine based on inhibition of hepatocyte invasion will probably be difficult.

Michael R. Hollingdale

*Malaria Department,
Biomedical Research Institute,
Rockville,
Maryland 20852, USA*

Robert E. Sinden

Jos van Pelt

*Imperial College of Science, Technology
and Medicine,
London SW7 2BB, UK*

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Missing link in ion channels

SIR — Na⁺ and Ca²⁺ channels comprise four homologous domains, each of which is thought to contain six transmembrane segments. K⁺ channels, on the other hand, have a single such domain, and appear to function as tetramers. Based on voltage-clamp data from phylogenetically diverse organisms and on sequence similarities of each domain of the Na⁺ channel to the corresponding domain of the Ca²⁺ channel, Hille¹ has proposed that an ancestral single-domain channel gene gave rise to a primordial four-domain Ca²⁺ channel by two successive intragenic duplications; subsequent gene duplication and divergence then gave rise to the first Na⁺ channel. Our phylogenetic analyses² support this scheme, which implies the historical existence of functional two-domain channels.

Two recent reports may illuminate these historical events. First, the pre-

dominant isoform of Ca²⁺ channel in newborn rabbit muscle appears to consist of a two-domain structure, seemingly the product of an alternate splicing event of a transcript derived from a four-domain gene³. Although Isacoff *et al.*⁴ have constructed and expressed a fusion protein consisting of two *Shaker* K⁺-channel domains, this is the first evidence for a naturally occurring channel with a two-domain structure, the product of a putative splice event resulting in the precise joining of homologous sequences in domains II and IV. Although the genomic structure has not yet been determined, this suggests that an intron was present at this position in the two-domain ancestor of the modern Ca²⁺ channel gene, evidence for which may yet be found in this or related genes.

Second, two recently identified *Drosophila* genes, *transient receptor potential* (*trp*) and *transient receptor potential-like* (*trpl*), encode putative Ca²⁺ channels involved in photoreception. Both proteins consist of a single domain of six putative transmembrane segments which show significant sequence similarity to known Ca²⁺ and Na⁺ channels^{5–7}. This single-domain channel may provide a missing link in the evolution of the ion channel superfamily, suggesting that the acquisition of Ca²⁺ selectivity preceded both of the duplication events which gave rise to the modern four-domain Ca²⁺ and Na⁺ channels. The question of whether K⁺ or Ca²⁺ selectivity represents the more ancient property of single-domain channels remains open.

Michael Strong

*Department of Physiology
and Biophysics,*

George A. Gutman

*Department of Microbiology
and Molecular Genetics,
University of California,
Irvine,
California 92717, USA*

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Scientific Correspondence

Scientific Correspondence is a relatively informal section of *Nature* in which matters of general scientific interest, not necessarily those arising from papers appearing in *Nature*, are published. Because there is space to print only a small proportion of the letters received, priority is usually given according to general interest and topicality, to contributions of fewer than 500 words, and to contributions using simple language.

Botanical lore

Ryan J. Huxtable

World Medicine: Plants, Patients and People. By David Bellamy and Andrea Pfister. Blackwell: 1992. Pp. 456. £16.99, \$24.95.

Murder, Magic and Medicine. By John Mann. Oxford University Press: 1992. Pp. 232. £16.95, \$29.95.

THESE alliterative titles seem designed more to catch the eye than to inform. One of the main questions addressed by Bellamy and Pfister is how the empirical folk knowledge of the properties of plants slowly transformed itself into mainstream Western medicine. We are taken on a historical tour of the plants used by the various cultures that have contributed to modern Western views of medicine. The marks these cultures have left are enduring. The medical papyri of Pharaonic Egypt date to 1500 BC and list henbane, hemp, linseed, mandrake, mint, poppy, senna, squill and thyme among the vegetable drugs, all of which are still to be found in modern herbals. The authors comment on the number of prescriptions containing honey as an ingredient, apparently unaware of the antibacterial potency of the material.

From the classic Talmudic period of the Jews come detailed laws on hygiene, diet and lifestyle, although our understanding of how these evolved is tainted by after-the-fact reasoning. It is, however, the Graeco-Roman tradition that has had the biggest influence on the evolution of Western medicine. The Hippocratic oath, although repeated by few, is familiar to all. The sheer size of the Roman Empire, which encompassed and was bounded by many cultures, allowed for facile interchange of information between East and West, from the Orient to the Mediterranean. Trade accompanied the spread of knowledge. The Romans obtained ebony from Sri Lanka and Mauritius, aloes from Africa and dragon's blood from Socotra, while giving the walnut, chestnut, plantago and stinging nettle to England. The myrrh and frankincense of the New Testament came from Arabian species of *Commiphora* and *Boswellia*, members of the Burseraceae. The Romans initiated the trade routes for Eastern spices that grew to such prominence in the economy of pre-modern Europe. Their contributions to public health can still be seen in the aqueducts scattered across Europe, and experienced in the sewers of ancient Rome which, the authors mordantly observe, are "still extant and at times functional".

Hellenic learning was preserved during the Dark Ages by the Arabs and, leavened with their own contributions, began to re-enter Europe via the School

of Salerno in the eleventh century. The *Regimen Sanitatis Salernitanum* was influential in disseminating this recovered learning. Arabic contributions included the art of distillation and the findings of the Persian philosopher and physician Ibn-Sina, known to the West as Avicenna.

A great influx of ethnobotanic knowledge came with the colonization of the New World. A helter-skelter mixture of diseases, plant foods and drugs crowded into Europe, including syphilis, guaiacum for its treatment, quinine-containing bark, beans, squash, sassafras, corn (maize), potatoes, tomatoes, tobacco and chocolate. Ben Jonson alludes to this New World cornucopia in his play *Volpone* (1606):

No Indian drug had e'er been famed,
Tobacco, sassafras not named;
Nor yet, of guacum one small stick, sir . . .

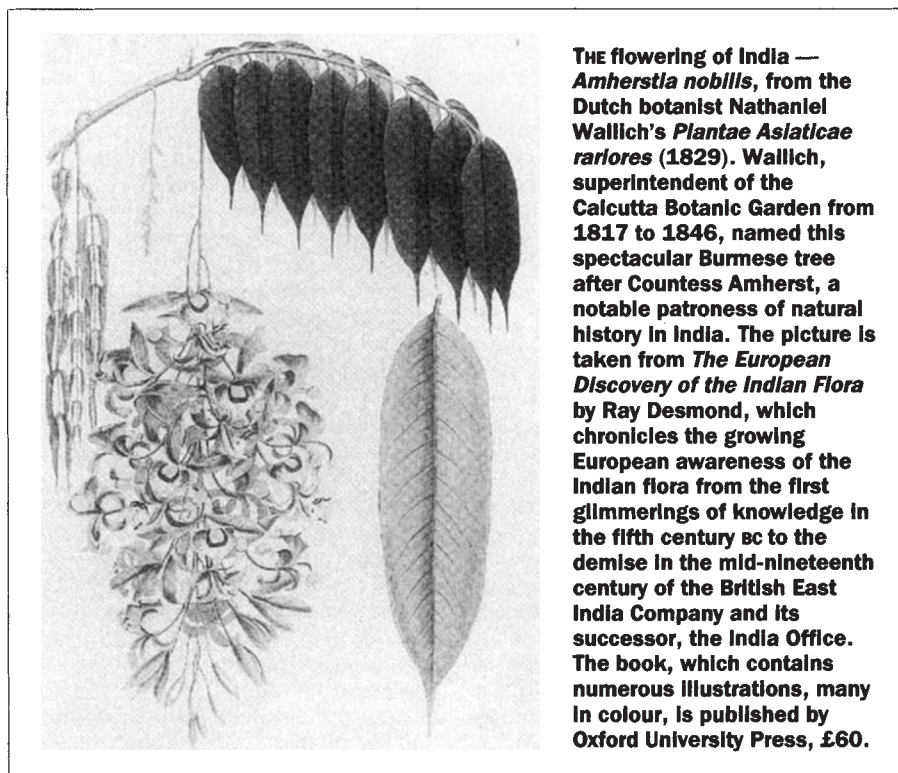
He goes on to describe tobacco in *Every Man in His Humour* as "good for nothing but to choke a man, and fill him full of smoke and embers".

The impact of the New World is still with us. The nineteenth century saw the

discovery of cocaine and mescaline. In this century, the Americas have given us emetine, the curare alkaloids and theobromine. Bellamy and Pfister even make the claim that the Mexican yam (*Dioscorea* species) is possibly history's most important plant because it yields the starting material for much of the world's supply of contraceptive steroids. Russell E. Marker's synthesis of progesterone from yam sapogenins is described as "the greatest landmark in the history of modern medicine".

In reading *World Medicine*, one is struck by the continuity of thought through the ages. Belief in the four elements of matter — earth, air, fire and water — persisted for centuries. Each gave rise to a 'humour' that conditioned both personality and health. Thus, phlegm derived from water, yellow bile from air, blood from fire and black bile from earth. These concepts are traceable to Thales of Miletus, who lived around 630 BC. Yet they still survive in language in the form of adverbs such as phlegmatic, sanguine, melancholy, humorous, bilious and jaundiced. Appropriate combinations of humours resulted in the characteristics of heat, dryness, coldness and moisture. Three of these four terms are still used to describe personality. Nowadays, we release hormones and transmitters rather than secretions, but maybe the change is more in the terminology than in the underlying thinking.

Bellamy is a botanist from Cheam in England, Pfister an ecologist from Rome. They have produced an admirable book, written in a colloquial style of



THE flowering of India —
Amherstia nobilis, from the Dutch botanist Nathaniel Wallich's *Plantae Asiaticae rariores* (1829). Wallich, superintendent of the Calcutta Botanic Garden from 1817 to 1846, named this spectacular Burmese tree after Countess Amherst, a notable patroness of natural history in India. The picture is taken from *The European Discovery of the Indian Flora* by Ray Desmond, which chronicles the growing European awareness of the Indian flora from the first glimmerings of knowledge in the fifth century BC to the demise in the mid-nineteenth century of the British East India Company and its successor, the India Office. The book, which contains numerous illustrations, many in colour, is published by Oxford University Press, £60.

'reallys' and 'wells' which, if not exactly to my taste, is preferable to the convoluted gobbledygook beloved of so many academic writers.

Even the presence of an appreciative quotation from my own writings in *Murder, Magic and Medicine* does not quiet the feeling that too close a knowledge of a book's topic is a disqualification for a reviewer. The sense of *déjà vu* — or *déjà lit* — colours one's response to a book in a way that perhaps it would not for a reader approaching the subject matter for the first time.

This book's title is a poor guide to its contents. In fact, the book is an overview of ethnopharmacology and the history of natural products, mixed in with brief explanations of nerve conduction, the structure of the heart, neuronal transmission and how the sodium channel and the menstrual cycle work. The text is leavened with canonical quotations from Shakespeare, De Quincey and Coleridge, with the occasional line of Latin to remind us how educational standards have regressed over the past century.

All the stories one expects to find are here, crammed in glorious propinquity: of how the mandrake root screams when disanchored from the earth, how the hunters of the Orinoco tip their arrows with curare, the gustatory risks of fugu, and how absinthe makes the heart grow fonder (or at least more amorous).

The book is an excellent introductory text for those not liable to dizziness as they jump from one culture to another, or one century to the next, and who are able to read about absinthe and kava on the same page without getting confused. It is ideal for high-school students and undergraduates with an interest in the colourful history of pharmacology and a desire to know what causes the growth of the mammary glands in females. Even pharmacologists raised in a scientific culture in which a two-year-old paper is out of date could spend an idle hour or two profitably browsing, and learning that their discipline has roots in more senses than one.

One problem with putting infinity under a bell jar is that detail is lost, and statements are made with a black-and-white certitude that belies reality. And when a book exhales information as a carpet does dust, it is an invitation to carping on the part of the reader. In the discussion of ackee fruit poisoning, there is no mention of the agents involved, the hypoglycins. The reader does not learn that the lethal tetrodotoxin contained within the fugu or puffer fish is made not by the fish itself, but is obtained from its diet, originating near the bottom of the food chain in bacteria. Acetylcholine, we are told, "is undoubtedly the most important neurotransmitter", leaving me

wondering what the criteria of importance are — there are certainly more abundant transmitters. And Napoleon's hair may contain arsenic, but hair readily picks up arsenic from the environment, including soil it may have contacted, so a conclusion that Napoleon consumed arsenic before dying needs tempering.

Many statements in the book invite scepticism. Do addicts really "often describe" crack cocaine as "an orgasm in every cell of one's body"? And where are these "many tribes in contemporary Africa" using extracts of *Datura* for the ceremonial deflowering of pubescent girls? As this example suggests, the book is written from a culturally normative

viewpoint. The author also talks about trial by ordeal in "primitive societies" (also in Africa), without mentioning that such trials were a feature of mediaeval Europe.

If this book encourages the reader to delve more deeply into the relationship between man and natural products, then it will have fulfilled its author's purpose. It is well produced and copiously illustrated. As a source book, however, it suffers from a lack of references. □

Ryan J. Huxtable is in the Department of Pharmacology, College of Medicine, University of Arizona, Tucson, Arizona 85724, USA.

Memories of the future

John D. Barrow

Einstein's Dreams. By Alan Lightman. Pantheon/Bloomsbury: 1993. Pp. 179. \$17, £13.99.

THERE'S not much scientific fiction that's not science fiction. And the best of it is generally an incorporation of particular scientific ideas within a literary setting. Thus one finds the anthropic principle as the centrepiece of a John Updike novel, Borges's short stories incorporating subtleties of time and infinity and less subtle novels about the human consequences of a reversal of time's arrow.

This little book is different. It is written by someone who is primarily a scientist. Although described as a novel, it is really a sequence of short cameos, each offering a vivid impression of a different dream world where the nature of time is deeply strange. In one it is circular, in another there are two times, in another the slowing of clocks in strong gravity fields has made everyone live in the mountains. In other worlds, time is erratic, or the soon expected end of time induces a weird fatalism. Elsewhere there are moving glimpses of a world in which time stands still, or where there is no time at all — merely images. And we visit a world without memory where passions are ever new; a world where time flows backward; and an ephemeral world where life is speeded up and lasts for a single day.

There are dream worlds where time is just an extra sense or where people live forever. This produces a strange division of the population into the "Lateres" and the "Nows". The former are the procrastinators who see in their unending futures ample time to do anything they choose. Thus all things can wait. The Nows see the potential to do an infinite number of things, all they can imagine: "they begin early and never go slowly".

But the sociology is curious. Society contains so much experience that it acts as a deadweight upon the accomplishment of anything. "Sons never escape from the shadows of their fathers. Nor do daughters of their mothers. No one ever comes into their own." Sons seek advice from their fathers who seek advice from their fathers and so on. Life is tentative. The unending sequence of verifications required before any project can be completed leads to a world of unfinished projects.

In another dream world, time is a quality not a quantity. Without clocks, calendars and appointments the world is driven by events, ripeness. Everything happens in the fullness of time. There are worlds without futures, worlds where time stops now and then, worlds where the inhabitants are enslaved by worship of the Great Clock; fragmented worlds where the flow of time differs from place to place; and worlds where people live in the past.

Lightman's writing has a telling brevity and precision. His stories are moving and poignant and the result is a unique perception of the ways in which the actual nature of time fashions human relationships and aspirations. This work does not feel like a book by a scientist. It reveals a clear and careful study of human nature. Its simplicity and thoughtfulness are reminiscent of Primo Levi. This is the best work of fiction by a scientist that I have read. It passes some of the tests of classic work: it provokes immediate rereading and a description of it cannot replace the experience of reading it. It's tantalizingly short but lives long in the memory. Take time to read it. □

John D. Barrow is in the Astronomy Centre, University of Sussex, Falmer, Brighton BN1 9QH, UK.

A son of genius

Maurice Crosland

Humphry Davy: Science and Power. By David Knight. Blackwell: 1992. Pp. 218. £30. \$29.95.

"DAVY is a wonderful subject for a biography because he is so accessible", writes David Knight, and this is true in more senses than one. Not only was he concerned with scientific communication

He was the son of a woodcarver, and his career began as apprentice to an apothecary before he abandoned this path to become the assistant of Thomas Beddoes at his Pneumatic Institute in Bristol. Here Davy's investigation of nitrous oxide was to bring him favourable attention and he was offered a post at the newly founded Royal Institution, which was to provide him with both an excellent laboratory and a platform for popular lectures to fashionable audiences. He was a very successful showman but he was also brilliant in his research. Since Berman's work (*Social Change and Scientific Organization*, Heinmann, 1978), Davy's agricultural research cannot be overlooked; but Davy is probably remembered best for his isolation of the alkali metals, using the new electric battery which had not been available to the generation of Lavoisier. Yet at the height of his fame as professor at the Royal Institution he resigned his position. Had he felt that his creative powers were waning? The answer is rather that he had in his sights a wealthy widow. When she accepted his proposal of marriage, he could look forward to a life of leisure and greater social esteem. He had already been a prominent figure on the London social scene. On 8 April 1812 he was knighted for his services to science. On 10 April he delivered his last lecture and on 11 April, as Sir Humphry, he married Mrs Apreece who became Lady Davy. All this from a man who

Mary Evans

in his youth had spoken in the poem above of the "sons of genius" who despise "wealth, power and grandeur".

Yet Davy continued to contribute spasmodically to science, and his successful consultancy led to his invention of the miners' safety lamp. In his new social position it was not appropriate that he should seek a patent. He was an obvious candidate for the presidency of the Royal Society when Sir Joseph Banks died in 1820. Knight shows that Davy's presidency was not a happy one, nor were the last years of his life, to which the author gives inordinate attention. I would have liked more information on Davy's research career, including his rivalry with his exact contemporary Gay-Lussac over the discovery of chlorine and iodine, but it could well be argued that these topics have already been adequately covered.

Knight has been passionately concerned with Davy for a quarter of a century and could easily have written a book three times the size. Instead, he has produced a brief but authoritative and most readable overview of Davy's life and work, giving enough references and bibliography for readers to follow up aspects that particularly interest them. He reveals Davy not as an impersonal scientist, but as a full human being. □

Maurice Crosland is in the History of Science Unit, Physics Laboratory, University of Kent, Canterbury, Kent CT2 7NR, UK.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Social climber: Davy (1778–1829).

and popularization, but much of his work was published, and for manuscript sources the researcher hardly has to go further than the Royal Institution in London. Davy has therefore naturally been the subject of many biographies, starting with that by J. A. Paris in 1831 which, because of some criticism contained in it, was followed by a further full biography by Davy's brother. More recently (1966), there was a biography by Sir Harold Hartley, who was exclusively interested in Davy's scientific work. Knight insists that this was only one aspect of Davy.

Knight reminds us that Davy was not only a chemist but a poet as well, and there are liberal quotations from Davy's verse throughout the book. Science and the Romantic imagination were related in such stanzas as:

To scan the laws of nature, to explore
The tranquil reign of mild Philosophy
Or on Nature's wings to soar
Through the bright regions of the starry sky.

Davy and Coleridge were close friends and clearly influenced each other. Davy also knew Wordsworth and managed to persuade him that there was a creative and imaginative side to science. Perhaps Knight performs a similar service for nonscientific readers. Yet he avoids presenting Davy simply as a hero.

Davy was very much a social climber.

Simple explanations

Michael Shlesinger

Applied Chaos Theory: A Paradigm for Complexity. By A. B. Çambel. Academic: 1993. Pp. 246. \$44.95, £33.

THERE have traditionally been two great trends in physics: investigating the very small (atomic, nuclear, particle physics) and investigating the very large (astronomy, cosmology). It is only in the past decade or so that a third trend has emerged: investigating the very complex. The best known examples are the overlapping topics of chaos and fractals, the recent success of which lies in understanding complex behaviour on the basis of simple laws. This fortunate turn of events allows one to learn a fair amount rapidly without great effort. The simplicity can be misleading, with the field being too easy for beginners and too hard for experts.

What should you read if you want to learn about the torrent of new ideas in the field of complexity? If you are an expert, there are several superb weighty mathematical physics tomes that demand

much effort and time. If you are not an expert, you could with great profit read James Gleick's bestseller *Chaos* (Viking, 1987), but this is more about scientists and their interactions than about science. *Applied Chaos Theory* provides one with an alternative. This short, somewhat conversational book is illuminating on a variety of topics, such as attractors, nonlinear population equations, logistic maps, fractal dimensions and Mandelbrot sets. It is written in an engaging manner at a level suitable for undergraduates, and the references are well chosen and fairly up to date.

Çambel sums up succinctly his rationale for the study of chaos: "The importance of chaos is . . . to tackle problems that in the past we simply shied away from." Limits to this approach exist. For example, the older field of fluid turbulence (the classic spatio-temporal dynamics problem) is still shied away from because it has not succumbed to simple arguments. Despite his wish to tackle problems and the word "Applied" in the book's title, Çambel's

focus is on philosophy and ideas, and scant attention is paid to applications. For example, he writes: "Chaos control is being studied in chemically reacting systems . . . Such research could lead to enhanced productivity in chemical processing and could be useful in improving chemical combustion." But not much more detail than references is given for possible applications. Chaos is strictly defined only for deterministic systems and is characterized by the behaviour of orbits. Again, important concepts distinct from chaos are referred to, including probability ideas, statistical mechanics and random fractals.

Chaos theory has relied heavily on computer graphics to follow dynamical orbits and has paid less attention to precise mathematical solutions. This, in part, is because one must accept the loss of analytical solutions for nonlinear equations where slight variations in a parameter can change the behaviour of a solution from periodic to chaotic and because general solutions with these properties have not been forthcoming. Çambel keeps his mathematics to a minimum, using it more as a caption than as a challenge for solution or manipulation. The discussion of entropy from its statistical mechanical origins, through information theory, to the measurement of divergence of dynamical orbits is illuminating and informative (although in the Rényi entropy equation on page 156, $p_i \log p_i$ should be replaced by p_i^q).

Several software packages are used to generate the figures in the book, such as the bifurcation diagram of the logistic map. This is perhaps the best known picture from chaos theory and it is not difficult to program. Bifurcation plots of other maps in the same universality class would have been useful. For example, a comparison of values where bifurcations occur in the sine map and the logistic map would demonstrate the power of universality when it applies.

Biology is one area where I would have liked a stronger emphasis: living organisms with their emergent properties based on the laws of physics are, perhaps, the ultimate complex system. New discoveries, such as control of biological oscillators (including cardiac tissue), stochastic resonance in the peripheral nervous system, spatio-temporal cortical voltage patterns and motor-sensory transient synchronization, are ripe for dynamical treatment.

The book ends with thought-provoking discussion topics and ideas for one's own home chaos laboratory. For the uninitiated, Çambel's book will be a rewarding experience. □

Michael Shlesinger is at the Office of Naval Research, 800 North Quincy Street, Arlington, Virginia 22217-5000, USA.

Biodiversity in brief

Norman Myers

Global Biodiversity: Status of the Earth's Living Resources. Edited by B. Groombridge. Chapman and Hall: 1992. Pp. 585. £30, \$59.95.

Conserving Biodiversity: A Research Agenda for Development Agencies. By the Board on Science and Technology for International Development, US National Research Council. National Academy Press: 1992. Pp. 127. \$19.00 (pbk).

Systematics, Ecology, and the Biodiversity Crisis. Edited by N. Eldredge. Columbia University Press: 1992. Pp. 220. \$61.50.

THE message that we are on the verge of a biotic holocaust is steadily dawning on the general public, and, less strongly, on our political leaders. So there is good cause to welcome three fine books that, in their disparate ways, help to present the message still more forcefully.

By far the best in terms of information and documentation is *Global Biodiversity*, compiled by the World Conservation Monitoring Centre, Cambridge, United Kingdom. It is a splendid compendium on biodiversity from the standpoint of systematics, genetic diversity, species-rich areas, habitat classification, resource economics, international conservation and a host of other topics. The encyclopaedic format is packed with data, making this a collation that is way ahead of anything else in the field so far. It is understandable though regrettable that there should be sundry errors of data — probably a reflection of the fact that the book is too selective in its professional sources. A number of leading experts are not even cited. It is curious too that the book is sometimes not so up to date as it might be generally. And, more particularly, it is unfair to criticize scientists for assertions made a dozen years ago while ignoring subsequent papers in which they have advanced their analyses.

A further limitation is the lack of any substantive assessment of the survival outlook for biodiversity. When the issue in question is the only environmental problem of our time that will leave its mark for many millions rather than a few hundred years, one would expect there to be an appraisal, however summary, of biodiversity's prospects. Nor is there anything on what will surely turn out to be more important in the long run than the loss of large numbers of species: the impoverishing effect on evolution for a period at least 200,000 times longer than

humankind has existed as a species. Or perhaps I have missed something during my perusal: the book has no index.

Conserving Biodiversity is a succinct report by an expert panel convened to address some pragmatic challenges of biodiversity conservation. It explicitly avoids a rehashing of biodiversity's importance and decline, nor does it aim for a comprehensive action plan or even a technical research programme. It examines several avenues of research that will enable development agencies to play a larger role in biodiversity conservation within a context of sustainable development. The book succeeds admirably with the first part, but not so well with the second (largely because nobody yet knows exactly what sustainable development means, especially in operational terms). It is soundest on project- and country-level initiatives, with some emphasis on local knowledge.

Systematics, Ecology, and the Biodiversity Crisis consists of symposium papers by leading systematists, taxonomists, palaeontologists and ecologists who explore the relationship between systematics and other biological sciences with respect to our "understanding of the origin, maintenance and loss of biological diversity". As Niles Eldredge asserts, "It may well be that the dynamics of extinction processes will prove to be . . . exclusively in the realm of ecology. But the problems of extinction can be defined, recognized, measured, and assessed only through the tools of the systematist."

His claim is well supported in most of the book. The analyses are generally probing and stimulating, drawing on geology, genealogy, phylogeny, palaeontology and population biology among other fields. Nor is the book unduly theoretical: there are stacks of case-studies from Madagascar, Cuba, Chile, East Africa and the oceans. An illuminating volume. □

Norman Myers, Upper Meadow, Old Road, Headington, Oxford OX3 8SZ, UK, is a consultant in environment and development.

■ The International Council for Bird Preservation (Cambridge, UK) has recently published *Putting Biodiversity on the Map* (£12.50, \$23; pbk), which aims to identify priority areas for the conservation of global biological diversity. It is based on a "thorough and rigorous" analysis of bird distributional data. Because they occur in most habitats on land and their taxonomy and distribution are better documented than for any other life form, birds make excellent indicators of biodiversity. The study reveals that about 20 per cent of all bird species are confined to just 2 per cent of the Earth's land surface and pinpoints 221 conservation 'hotspots'.

Cloning and expression of an inwardly rectifying ATP-regulated potassium channel

Kevin Ho*, Colin G. Nichols†, W. Jonathan Lederer‡, Jonathan Lytton*, Peter M. Vassilev§, Marie V. Kanazirska§ & Steven C. Hebert*||

* Harvard Center for the Study of Kidney Disease, Harvard Medical School and Laboratory of Molecular Physiology and Biophysics, Renal Division, Department of Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, Massachusetts 02115, USA

† Department of Cell Biology and Physiology, Washington University School of Medicine, Washington University Medical Center, St Louis, Missouri 63110, USA

‡ Department of Physiology, University of Maryland, Baltimore, Maryland 21201, USA

§ Endocrine and Hypertension Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115, USA

A complementary DNA encoding an ATP-regulated potassium channel has been isolated by expression cloning from rat kidney. The predicted 45K protein, which features two potential membrane-spanning helices and a proposed ATP-binding domain, represents a major departure from the basic structural design characteristic of voltage-gated and second messenger-gated ion channels. But the presence of an H5 region, which is likely to form the ion conduction pathway, indicates that the protein may share a common origin with voltage-gated potassium channel proteins.

POTASSIUM channels, which are ubiquitous in eukaryotic cells, exhibit an exceptional functional diversity, making them ideally suited for their roles in a broad spectrum of processes in excitable and non-excitable cells. An understanding of the molecular basis for this diversity has emerged in the case of voltage-gated K^+ channels¹ with the cloning and characterization of the *Shaker* gene in *Drosophila melanogaster*²⁻⁵. Alternative splicing of the *Shaker* primary transcript^{3,6-8} generates a family of structurally related proteins sharing a core of transmembrane domains flanked by variable N- and C-terminal regions. Expression of these proteins in *Xenopus* oocytes yields voltage-gated K^+ channels with distinct biophysical and pharmacological properties^{9,10}. Additional functional and structural heterogeneity is provided by *Shal*, *Shab* and *Shaw*, which together with *Shaker* comprise an extended gene family coding for rapidly inactivating A-type and delayed rectifier-type channels¹¹. The cloning of numerous mammalian homologues corresponding to each of these genes has defined four subfamilies¹²⁻²⁰. Unlike their invertebrate counterparts, mammalian channel proteins are generally products of single uninterrupted exons²¹. Diversity arises primarily from a multiplicity of intronless genes instead of from alternative splicing²². Within the conserved regions, S1-S6, the amino-acid identity shared by *Drosophila* and mammalian homologues from a subfamily (55-82%) exceeds that present among *Drosophila* proteins from different subfamilies (38-42%). This supports the notion that the *Shaker*, *Shal*, *Shab* and *Shaw* subfamilies arose before the divergence of vertebrate and invertebrate species¹². Moreover, the recent discovery of homologous K^+ channel genes in the plant *Arabidopsis thaliana*^{23,24} dates the hypothetical ancestral gene for the *Shaker* family to the Precambrian era, before the evolution of the eukaryotic lineages Plantae, Fungi and Animalia²⁵.

Voltage-gated K^+ channels belong to the superfamily of voltage-gated and second messenger-gated cation channels^{25,26} which encompasses voltage-dependent Na^+ and Ca^{2+} channels²⁷, cyclic nucleotide-gated channels^{29,30} and *Slo* Ca^{2+} -activated K^+ channels^{31,32}. In addition to sequence similarity, polypeptides in this superfamily contain one or four copies of an underlying structural motif characterized by six membrane-spanning segments (S1-S6), a putative voltage sensor (S4 seg-

ment), and an S5-S6 linker (H5 region) involved in ion conduction. All K^+ channel proteins cloned so far, with the exception of the minK ($I_{K(M)}$) protein³³, share a similar structural organization based on one such motif, thus establishing their kinship to this ion-channel superfamily. However, the molecular features of many other K^+ channel types (including ATP-sensitive, muscarinic-activated, S type, SK Ca^{2+} -activated, Na^+ -activated, and inward rectifier type)²⁸ are unknown.

We report here the deduced amino-acid sequence and novel structural features of a K^+ channel polypeptide, ROMK1, encoded by a cDNA cloned from mammalian kidney. Expression of the ROMK1 protein in *Xenopus* oocytes gives rise to an inwardly rectifying, ATP-regulated K^+ channel. An analysis of the protein's primary structure reveals a unique molecular architecture. In contrast to channel proteins belonging to the superfamily of voltage-gated and second messenger-gated ion channels, the ROMK1 protein does not have the canonical structural motif of six membrane-spanning helices nor of a typical S4 segment. But the protein conserves an amino-acid segment that is homologous to the pore-forming H5 region of voltage-gated K^+ channels, providing further evidence that the H5 region forms the ion permeation pathway. In addition, a single putative ATP-binding site which is associated with a cluster of potential phosphorylation sites and basic amino acids may form a regulatory domain.

Cloning and expression of cDNA

Xenopus laevis oocytes injected with poly(A)⁺ RNA isolated from the inner stripe of outer medulla of rat kidneys exhibited inward Ba^{2+} -sensitive currents ($I_{K(Ba)}$) (Fig. 1a, b) which were carried selectively by K^+ (Fig. 1c). Oocytes injected with size-fractionated mRNA of ~2-3 kb displayed an $I_{K(Ba)}$ 16-fold greater than that in H_2O -injected controls (Fig. 1d). This mRNA size fraction was subsequently used to construct a directional cDNA library. Functional screening of cRNA transcribed *in vitro* from pooled clones led to the identification of a 2.1-kb cDNA, ROMK1.

ROMK1 cRNA-injected oocytes (Fig. 1e) gave an $I_{K(Ba)}$ of $-1,408 \pm 179$ nA ($n = 10$, $V_m = -60$ mV, $[K^+]_o = 50$ mM). In contrast to Ba^{2+} , 10 mM TEA⁺ had no effect on whole-cell currents ($n = 8$). Characterization of these currents in ion substitution experiments (substituting Na^+ for external K^+) demonstrated that the expressed channels were highly selective for K^+ (Fig. 1f).

|| To whom correspondence should be addressed.

Characterization of ROMK1 channels

Single-channel recordings were obtained from ROMK1 cRNA-injected oocytes in order to characterize the expressed channels mediating $I_{K(Ba)}$. Membrane patches from cRNA-injected oocytes contained a high density of intermediate-conductance channels (current tracings shown in Fig. 2a) that were never observed in H₂O-injected controls. In inside-out patches, single-channel currents exhibited inward rectification in the presence of Mg²⁺ (Fig. 2a, c). The unitary slope conductance for inward currents was 39 pS in symmetrical 145 mM K⁺ solutions. Single channel conductance (g) was found to be proportional to external K⁺ concentration ($[K^+]_o$) according to the relationship $g = 4.2 \times [K^+]_o^{0.44}$. This is similar to analyses³⁴ that approximate the K⁺ conductance (g_K) of inward rectifiers as a square-root function of $[K^+]_o$. Consistent with findings for whole-cell Ba²⁺-sensitive currents ($I_{K(Ba)}$) (Fig. 1f), single-channel currents were carried selectively by K⁺ ions (Fig. 2c). The open probability (P_o) of the expressed channels increased with depolarization, resulting in high channel activity at membrane potentials in the physiological range ($P_o = 0.82 \pm 0.02$ at -60 mV, 0.92 ± 0.01 at -30 mV). P_o was substantially reduced only at potentials more negative than about -60 mV (Fig. 2a, e).

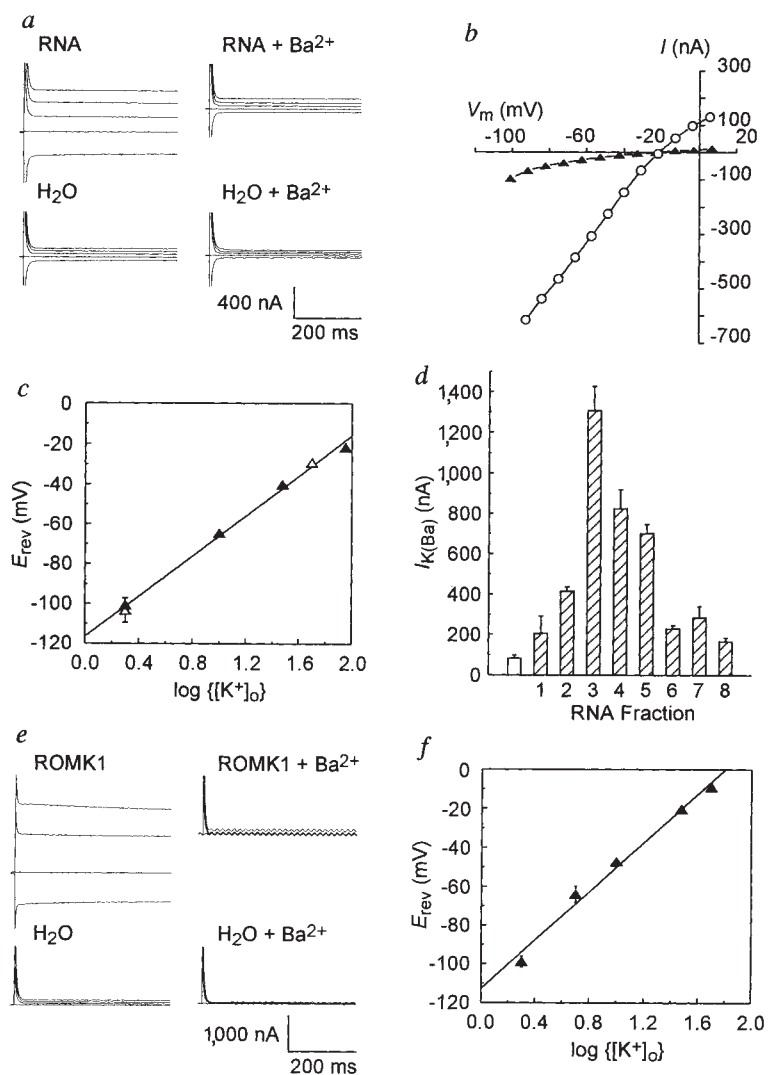
The effect of external Ba²⁺ ($[Ba^{2+}]_o$) on channels in inside-out patches was examined. In two-electrode voltage clamp experi-

ments, inhibition of inward currents by external 0.1 mM Ba²⁺ was dependent on membrane potential; the degree of block was augmented by increasing hyperpolarization (Fig. 2d). In patch clamp experiments, ROMK1 channel open probability was reduced in a voltage-dependent manner without an effect on single-channel current (Fig. 2b, e). With 0.1 mM external Ba²⁺, the fractions of unblocked macroscopic current (I_{Ba}/I_o) and of single channel P_o ($P_{o(Ba)}/P_o$) as a function of voltage were similar (Fig. 2f), confirming that the channels mediated whole-cell $I_{K(Ba)}$ currents.

Channel activity decreased spontaneously after patch excision (channel run-down) with a variable time course, as reported for other ion channels³⁵. Although the mechanism(s) underlying this phenomenon is not well defined, evidence suggests that patch excision (or whole-cell perfusion) results in the loss of one or more components promoting protein phosphorylation³⁵⁻³⁷. We therefore tested the effect of Mg-ATP on channels in inside-out patches. In about 20% of patches (7/35), partial or complete reactivation of channel activity following run-down was obtained by the addition of Mg-ATP (2.5-5.0 mM) to bath solutions. As illustrated in Fig. 2g, this effect of Mg-ATP required the continual presence of the nucleotide and was thus reversible; subsequent additions restored channel openings following a loss of channel activity in ATP-free solution. But it

FIG. 1 Ba²⁺-sensitive K⁺ currents ($I_{K(Ba)}$) in *Xenopus* oocytes injected with rat kidney inner stripe of outer medulla (ISOM) poly(A)⁺RNA or ROMK1 cRNA. a, Current tracings elicited by voltage steps from -80 to 0 mV in 20 mV increments (holding potential, $V_h = -60$ mV) in an ISOM poly(A)⁺ RNA or H₂O-injected oocyte. Ba²⁺ (5 mM)⁹² inhibited expressed currents in the RNA-injected oocyte. b, I - V relationships of $I_{K(Ba)}$ in an ISOM mRNA-injected (○) and a water-injected oocyte (▲). RNA-injected oocytes showed $I_{K(Ba)} = -335 \pm 19$ nA ($n = 31$), in comparison to -86 ± 8 nA for H₂O-injected controls ($n = 28$) at -75 mV. c, External K⁺-dependence of reversal potentials (E_{rev}) for $I_{K(Ba)}$ in mRNA-injected oocytes. External K⁺ ($[K^+]_o$) was replaced with NMDG⁺ (N-methyl-D-glucamine) (▲, $n = 7$, $[K^+] + [NMDG^+] = 98$ mM) or with Na⁺ (△, $n = 4$, $[K^+] + [Na^+] = 98$ mM) in perfusion solutions containing (mM) 0.3 CaCl₂, 1 MgCl₂, 5 HEPES (pH 7.6). E_{rev} (error bars, s.e.m.) varied linearly with $\log \{[K^+]_o\}$ (slope = 50 mV per 10-fold change in $[K^+]_o$). d, $I_{K(Ba)}$ in oocytes injected with ISOM mRNA size fractions 1-8 (~ 2 -4 kb) (hatched bars) and in H₂O-injected controls (open bar); bars represent mean $I_{K(Ba)}$ for 3-12 oocytes (error bars, s.e.m.). Oocytes injected with fraction 3 (~ 2 -3 kb) exhibited $I_{K(Ba)} = -1,307$ nA ($n = 4$, $V_h = -50$ mV) at -75 mV; for H₂O-injected controls, $I_{K(Ba)} = -83 \pm 14$ nA ($n = 8$). e, Current tracings in a ROMK1 cRNA ($V_h = -45$ mV) or H₂O-injected oocyte elicited by voltage steps from -60 to 0 mV in 20 mV increments. ROMK1 currents were inhibited by 10 mM Ba²⁺. f, ROMK1 $I_{K(Ba)}$ currents exhibited high K⁺-selectivity. External K⁺ was replaced by Na⁺ ($n = 5$, $[K^+] + [Na^+] = 98$ mM) in perfusion solutions containing (mM) 0.3 CaCl₂, 1 MgCl₂, 5 HEPES (pH 8.0). The E_{rev} of ROMK1 $I_{K(Ba)}$ currents was $[K^+]_o$ -dependent (slope = 63 mV per 10-fold change in $[K^+]_o$).

METHODS. ISOM mRNA was isolated and size-fractionated by sucrose gradient centrifugation⁹³. Double-stranded cDNA was prepared⁹⁴ from mRNA fractions 3 and 4 (d) using SuperScript Plasmid System (BRL). cDNA of 2-3 kb was ligated into pSPORT1 (BRL) and electroporated into DH10B cells (BRL) yielding $\sim 3.5 \times 10^5$ clones. *In vitro*-transcribed cRNA was prepared from pools of ~ 600 clones, injected into *Xenopus* oocytes and assayed for $I_{K(Ba)}$ by two-electrode voltage clamping. Clones from one positive pool were subdivided until the ROMK1 clone was identified. Currents were recorded by two-electrode voltage clamp (Axoclamp-2A (Axon Instruments)) from defolliculated oocytes injected 2-4 days earlier¹⁷ with ~ 50 nl ISOM mRNA ($0.5 \mu\text{g } \mu\text{l}^{-1}$), *in vitro*-transcribed cRNA ($\sim 7.5 \text{ ng } \mu\text{l}^{-1}$ ROMK1 cRNA), or H₂O. Currents were elicited by 450 ms test potentials from -100 to $+10$ mV in 10 -mV increments from the indicated V_h ($V_h = -60$ mV for RNA(+Ba²⁺) and H₂O-injected oocytes). $I_{K(Ba)}$ amplitudes were obtained by determining the difference in current amplitudes before and after Ba²⁺ addition and plotted



versus observed V_m . Recordings were acquired at 22 - 25°C from oocytes perfused with bath solution containing (in mM): 48 NaCl, 50 KCl, 0.3 CaCl₂, 1 MgCl₂, 5 HEPES ± 5 or 10 Ba²⁺ (pH 8.0). Microelectrodes (0.5 - 2 M Ω) contained 3.0 M KCl.

was observed that this channel-activating effect of Mg-ATP attenuated over time with readditions of the nucleotide. Channel run-down still occurred in the presence of Mg-ATP (25 μ M–5.0 mM), albeit at a slower rate than in its complete absence, suggesting a requirement for other factors necessary in maintaining channel function.

In kidney, ATP-sensitive (K_{ATP}) channels^{38,39} appear to predominate in epithelial cells of the outer medullary renal thick ascending limb and cortical collecting duct^{40,41}. These epithelial channels display properties (22–60 pS conductance, inward rectification, K^+ selectivity, high P_o , channel run-down, reactivation by 100 μ M Mg-ATP) comparable to those of ROMK1 channels expressed in oocytes, but renal K_{ATP} channels are inhibited by 1 mM Mg-ATP. Further work will be required to identify the exact physiological counterpart(s) for the ROMK1 channel and to ascertain whether the channel *in vivo* includes additional regulatory subunits or components.

Structural features

Sequencing of the 2,069-base-pair (bp) ROMK1 cDNA yielded a 1,173-bp open reading frame which is preceded by three in-frame termination codons and encodes a protein of 391 amino acids (M_r 45,000 (45K)) (Fig. 3a). The initiation codon was assigned to the first in-frame ATG which is contained within a strong Kozak consensus sequence⁴², AGCAUGG. The 3' untranslated region contains two polyadenylation signal sequences⁴³, ATTAATA, occurring 20 and 51 bases upstream from a poly(A) tail. The size of the putative polypeptide was confirmed by *in vitro* translation of ROMK1 cRNA, which yielded a ~41K product (Fig. 3b). A ~45K glycosylated product obtained by

translation in the presence of canine pancreatic microsomes (Fig. 3b, c) is in agreement with the single glycosylation site predicted for the putative protein. Transcripts of ~2.2, 3.2, 6, 9 and 12 kilobases (kb) were detected by northern blot analysis of rat kidney total RNA with renal outer medulla RNA yielding the most intense hybridization signals (Fig. 3d). Faint bands of ~3.7–4.2 kb were also detected on blots of RNA from several other tissues. It is unclear at present whether or not all of these species encode identical polypeptides.

Sequence comparison against the GenBank, EMBL and SWISS-PROT databases revealed no significant overall similarities. In fact, hydropathy analysis suggests a strikingly unique topology for ROMK1 unlike any ion channel protein cloned so far. Only two major hydrophobic peaks likely to represent membrane-spanning helices, M1 and M2, are apparent in ROMK1. This is in contrast to the hydropathy plots of K^+ channel proteins belonging to the superfamily of voltage-gated and second messenger-gated channels^{25,26} (Fig. 4), which share a basic structural motif of six potential transmembrane helices (S1–S6). Second messenger-gated channels also contain a single motif, whereas voltage-gated Na^+ and Ca^{2+} channels have four such motifs comprising homologous domains I to IV.

In spite of its novel topology, the ROMK1 protein contains an H5-like region (Fig. 3a), which is clearly related to the pore-forming H5 regions⁴⁴ of voltage-gated K^+ channel proteins. Comparison of ROMK1 and *Shaker* K^+ channel H5 sequences (Fig. 5a) reveals a similarity of 44% (28% identity, 16% conserved substitutions) for this segment of 25 amino acids. Within this region the greatest degree of similarity (59%: 41% identity, 18% conserved amino acids) occurs in a 17-amino-acid segment

FIG. 2 Single channel currents were recorded from inside-out patches excised from ROMK1 cRNA-injected oocytes. *a* and *b*, Current tracings from patches containing three active channels from the same oocyte recorded in symmetrical 145 mM K^+ solutions at various membrane potentials (V_m) with no Ba^{2+} (*a*) or 0.1 mM Ba^{2+} in the pipette solution (*b*). Downward deflections represent inward currents; closed and open channel current levels are indicated (long and short bars, respectively). *c*, Unitary current amplitudes derived from amplitude histograms plotted against membrane potential. Single channel recordings were obtained with pipette solutions containing 75 mM K^+ + 70 mM Na^+ ($\blacktriangle$) or 285 mM K^+ ($\triangle$); the calculated unitary slope conductances of inward currents were 31 pS and 47 pS, respectively (assuming linearity below E_{rev}). *d*, I – V relationships of macroscopic currents obtained by two-electrode voltage clamp in external solution (64 mM NaCl, 34 mM KCl, 1 mM $MgCl_2$, 5 mM Na-HEPES, pH 7.4) with ($\circ$; $n=4$) and without 0.1 mM Ba^{2+} ($\bullet$; $n=4$) using a 4-s voltage ramp from –120 to +120 mV; $V_{H_2O}=0$ mV. *e*, Voltage-dependence of channel open probability (P_o) with pipette solutions containing no Ba^{2+} ($\bullet$, mean $\pm$ s.e. (error bars), $n=4$) or 0.1 mM Ba^{2+} ($\circ$; $n=4$). *f*, Voltage-dependence of fractional single-channel open probability ($P_{o(Ba)}/P_o$ or ratio of single-channel open probability in the presence and absence of 0.1 mM Ba^{2+}) ($\bullet$) compared with fraction of unblocked macroscopic current (I_{Ba}/I_o or ratio of current with and without 0.1 mM Ba^{2+}) as a function of voltage ($\triangle$). Data are from *d* and *e*. *g*, Reversible activation of three channels in an inside-out patch by Mg-ATP. Channel activity was evident in the presence of bath solution containing 2.5 mM Mg-ATP but showed run-down in ATP-free bath solution. Channel openings could be restored by re-exposure to Mg-ATP. Labelled bars indicate bath solution changes; white bars below tracing indicate passage of patch pipette tip through oil gate. $V_m = -60$ mV. METHODS. Conventional patch clamp techniques⁹⁵ were used to record single channel currents from inside-out patches excised from ROMK1 cRNA-injected (~50 nl, 10 ng μ l⁻¹) *Xenopus* oocytes⁹⁶. Standard pipette solution contained (mM): 140 KCl, 1 $MgCl_2$, 5 or 10 K-HEPES, 1 K-EGTA, with or without 0.1 mM $BaCl_2$ (pH 7.4). Standard bath solution contained (mM): 140 KCl, 1 $MgCl_2$, 1 K-EGTA, 10 K-HEPES, 100 μ M ATP (Mg^{2+} salt). In *c*, the 75 mM K^+ pipette solution was prepared by mixing standard pipette solution with the following solution (mM): 5 KCl, 135 NaCl, 1 $MgCl_2$, 5 Na-HEPES (pH 7.4); the 285 mM K^+ pipette solution consisted of standard pipette solution with an additional 140 mM KCl. In *g*, the pipette solution differed from the standard solution by containing 98 mM KCl, and standard bath solutions with and without 2.5 mM ATP (Mg^{2+} salt) were used. Rapid solution changes were achieved using an oil-gate concentration jump technique⁹⁷. Details of single channel recording, data collection, and analysis have been described previously⁹⁸.

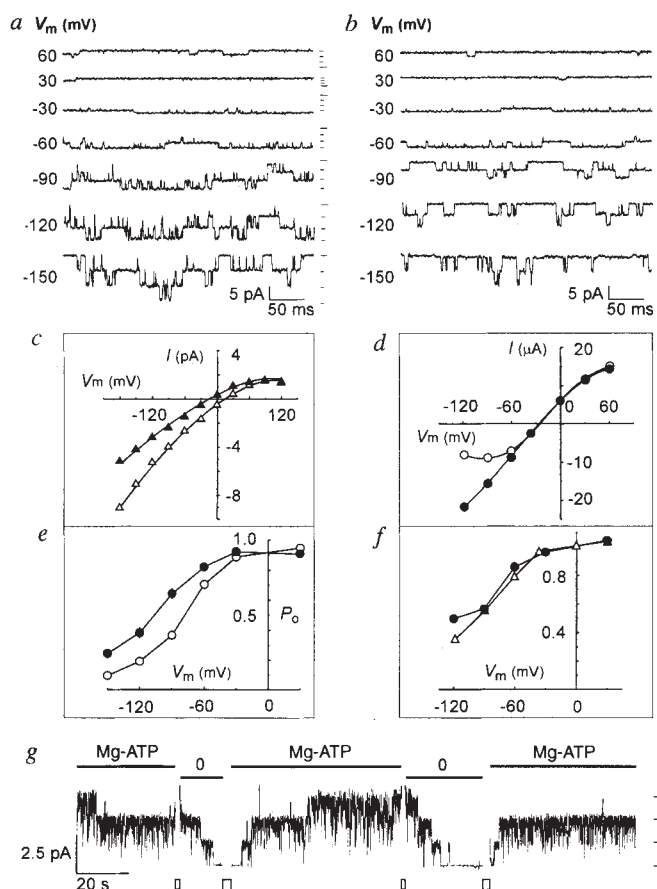


FIG. 3 *a*, Nucleotide and predicted amino-acid sequences of ROMK1 channel cDNA. Nucleotides are numbered beginning with the first position of the ATG initiation triplet; nonsense codons precede this ATG in all three frames. Proposed membrane-spanning segments, M1 and M2, are underlined as are the H5 and M0 regions. The single Walker type-A motif is double-underlined. Potential phosphorylation sites based on consensus motifs⁶² for protein kinase C (PKC) (●) and for cAMP-dependent protein kinase (PKA) (○) or possibly PKC (○) are labelled. Asn 117 occurs within the only *N*-linked glycosylation consensus sequence (arrow). Two polyadenylation signal sequences precede the poly(A) tail (dashed lines). *b*, in lane 1, *in vitro* translation of ROMK1 cRNA yielded a ~41K protein; a ~45K product was obtained by translation in the presence of canine pancreatic microsomes (M) (lane 2). No exogenous RNA was added in lanes 3 and 4. *c*, Endoglycosidase H (Endo H) treatment of the ~45K product in lane 3 resulted in a ~41K protein (lane 4) consistent with glycosylation of the ROMK1 protein; no effect was seen on nonglycosylated product (lane 2), *S. cerevisiae* α -factor mRNA was used as a control for translation (lane 5), glycosylation (lane 7), and Endo H treatment (lanes 6, 8). *d*, Northern blot analysis of total RNA (10 μ g) from rat kidney cortex, outer medulla and inner medulla (lanes 1, 2, 3), spleen (lane 4), lung (lane 5), eye (lane 6), thalamus (lane 7), hypothalamus (lane 8), and pituitary (lane 9) using a cRNA probe. No signal was detectable using RNA from cerebral cortex, hippocampus, cerebellum, brainstem, heart, aorta, skeletal muscle, esophagus, stomach, intestine, liver and adrenal.

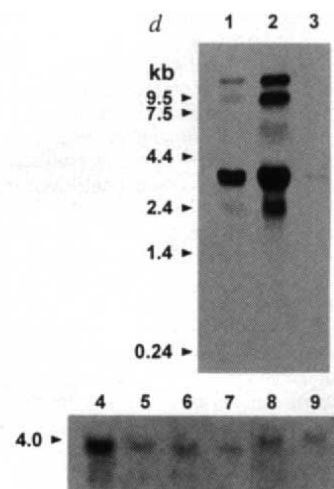
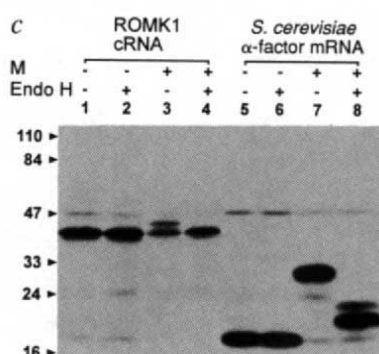
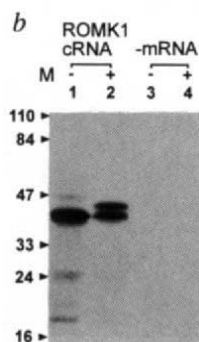
METHODS. Both strands of the ROMK1 cDNA were sequenced⁸⁹ using Sequenase Version 2.0 T7 DNA polymerase (US Biochemical). Geneworks 2.0 (IntelliGenetics) was used for sequence assembly and analysis. ROMK1 cRNA was translated *in vitro* using rabbit reticulocyte lysate and ³⁵S-methionine in the presence and absence of canine pancreatic microsomes (Promega). *S. cerevisiae* α -factor mRNA, *E. coli* β -lactamase mRNA and Brome mosaic virus RNA (Promega) were used as translation controls. Protein products were resolved by Laemmli SDS-polyacrylamide gel electrophoresis. For northern blots, membranes were hybridized (at 65 °C 50% formamide, 5 $\times$ SSCP, 0.1% Sarkosyl, 2% SDS) with a digoxigenin-labelled (Boehringer-Mannheim) full-length antisense cRNA probe transcribed from ROMK1 cDNA and washed at high stringency (65 °C, 0.1 $\times$ SSCP, 0.1% SDS). Hybridized probe was detected by chemiluminescence (Genius System, Boehringer Mannheim); exposure time was 30 min for lanes 1–3 and 16.5 h for lanes 4–9.

a

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-149 5'-CA ATC ACA CAA CTC CAC TCG AGT TAG CCA -121
TTG AAA GCC AAT GCA AGT AAA TGT CAT TCC AAA GCT TAA GAT TCA TTA AGG TGG GCC TAA -61
AAG AAG ACA GCT GCT GTG CAG ACA ACG TCG AAC AAG CAC CAC TTG CTT GCT TTG CCC AGC -1
Met Gly Ala Ser Glu Arg Ser Val Phe Arg Val Leu Ile Arg Ala Leu Thr Glu Arg Met 20
ATG GGC GCT TCG GAA CGG AGT GTG TTC AGA GTG CTG ATC AGG GCA CTG ACA GAA AGG ATG 60
Phe Lys His Leu Arg Arg Trp Phe Ile Thr His Ile Phe Gly Arg Ser Arg Gln Arg Ala 40
TTC AAA CAC CTC CGA AGA TGG TTT ATC ACT CAC ATA TTT GGG CGT TCC CGG CAA CGG GCA 120
Arg Leu Val Ser Lys Glu Gly Arg Cys Asn Ile Glu Phe Gly Asn Val Asp Ala Gln Ser 60
AGG CTG GTC TCT AAA GAA GGA AGA TGT AAC ATC GAG TTT GGC AAT CTG GAT CCA CAG TCA 180
Arg Phe Ile Phe Phe Val Asp Ile Trp Thr Thr Val Leu Asp Leu Lys Trp Arg Tyr Lys 80
AGG TTT ATA TTC TTT GTG GAC ATC TGG ACA ACT GTG CTG GAC CTG AAA TGG AGG TAC AAA 240
Met Thr Val Phe Ile Thr Ala Phe Leu Gly Ser Trp Phe Leu Phe Gly Leu Leu Trp Tyr 100
ATG ACC GTG TTC ATC ACA GGC ATG ATC ATC AAT TCG TTT CTG CTG TTT GGT GTC ATT TTA GCG 300
Val Val Ala Tyr Val His Lys Asp Leu Pro Glu Phe Thr Pro Pro Asp Asn Arg Thr Pro 120
GTC GTA GCG TAT GTT CAT AAG GAC CTC CCA GAG TTC TAC CCG CCT GAC AAC GCG ACT CCT 360
Cys Val Glu Asn Ile Asn Gly Met Thr Ser Ala Phe Thr Phe Ser Leu Glu Thr Gln Val 140
TGT GTG GAG AAC ATT AAT GGC ATG ACT TCA GCC TTT TCT TTT TCT TCT TCT TCT TCT TCT 420
Thr Ile Gly Tyr Gly Phe Arg Phe Val Thr Glu Gln Cys Ala Thr Ala Ile Phe Leu Leu 160
ACC ATA GGT TAC GGA TTC AGG TTT GTG ACA GAA CAG TGC GCC ACT GCG ATT TTC CTG CTT 480
Ile Phe Gln Ser Ile Leu Gly Val Ile Ile Asn Ser Phe Met Cys Gly Ala Ile Leu Ala 180
ATC TTC CAG TCT ATT CTT GGA ATC ATC AAT TCG ATG TGT GGT GTC ATT TTA GCG 540
Lys Ile Ser Arg Pro Lys Lys Arg Ala Lys Thr Ile Thr Phe Ser Lys Asn Ala Val Ile 200
AAG ATC TCT AGA CCC AAA AAA CGT GCT AAA ACC ATT AGC TTC AGC AAG AAT GCG GTG ATC 600
Ser Lys Arg Gly Gly Lys Lys Lys Leu Leu Ile Arg Val Ala Asn Leu Arg Lys Ser Leu 220
AGC AAG CGT GGC GGG AAG CTC TGC CTC ATC CGA GTG GCC AAT CTT AGG AAG AGC CTT 660
Leu Ile Gly Ser His Ile Tyr Gly Lys Leu Leu Lys Thr Thr Thr Thr Thr Thr Thr Thr 240
CTG ATT GGC AGC CAC ATA TAT GGC AAG CTT CTA AAG ACA ACC ATC ACT CCT GAA GGC GAG 720
Thr Ile Ile Leu Asp Gln Thr Asn Ile Asn Phe Val Val Asp Ala Gly Asn Leu Asn Leu 260
ACC ATC ATT TTG GAT CAG ACC AAC ATC AAC TTT GTC GTC GAC GCT GGC AAT GAA AAT TTG 780
Phe Phe Ile Ser Pro Leu Thr Ile Tyr His Ile Ile Asp His Asn Ser Pro Phe Phe His 280
TTC TTC ATA TCC CCA CTG ACG ATC TAC CAC ATT ATT GAC CAC AAC AGC CCT TTC TTC CAC 840
Met Ala Ala Glu Thr Leu Ser Gln Gln Asp Phe Glu Leu Val Val Phe Leu Asp Gly Thr 300
ATG GCA GCA GAA ACT CTT TCC CAA CAG GAC TTT GAG CTG GTG GTC TTT TTA GAT GGC ACA 900
Val Glu Ser Thr Ser Ala Thr Cys Gln Val Arg Thr Ser Tyr Val Pro Glu Glu Val Leu 320
GTG GAA TCC ACC AGT GCA ACC TGC CAG GTC CGC ACG TCA TAC GTC CCA GAG GAG GTG CTT 960
Trp Gly Tyr Arg Phe Val Pro Ile Val Ser Lys Thr Lys Glu Gly Lys Tyr Arg Val Asp 340
TGG GGC TAC CGT TTC GTT CTT ATT GTG TCC AAG ACC AAG GAA GGG AAA TAC CGA GTT GAT 1020
Phe His Asn Phe Gly Lys Thr Val Glu Val Glu Thr Pro His Cys Ala Met Cys Leu Tyr 360
TTT CAT AAC TTC GGT AAG ACA GTG GAA GTG GAG ACC CCT CAC TGT GCC ATG TGC CTC TAT 1080
Asn Glu Lys Asp Ala Arg Ala Arg Met Lys Arg Gly Tyr Asp Asn Pro Lys Phe Val Leu 380
AAT GAG AAA GAT GCC AGG GCC AGG ATG AAG AGA GGC TAT GAC AAC CCT AAC TTT GTC TTG 1140
Ser Glu Val Asp Glu Thr Asp Asp Thr Gln Met 391
TCA GAA GTT GAT GAA ACG GAG GAC ACC CAG ATG TAG CAG TGG CTT TTC CAC CTA CAA AAA 1200
GCC TCC CAA GGA CAT AAG GGT TGA CTG TGT TCA GAA GCA TCT GAC GGG GGT CTG AAA GCA 1260
GGA TGA GAA CAT GCT AAA TGT GCT AGC ACA GTC ACC CCT GAA CCC CAG GGC TAT GGT TCT 1320
ACA AGA CAC ATA GCT CTA TAA GGC TGC ATA CGS TGC ATG CAT GTG AAT GAA ACT GTG GAA 1380
GCC AAA GGG GCC CAC TTG GAT CCT CAC TAT GAC TGT GTA AGC TCA TAT CGT GTT GAT GGA 1440
AAC AAA GTC ATT CAA GGA CAA AAC TTA GGA GCT TTA GAA AGC TTC AGG AAC TAG CCA CAT 1500
TTC CTG TTT GAT TCT ATG GAT GAT GAG AAA GAT GCT TTT ATC TTA AAG TAG ACT TCT ATC 1560
AAT GGA AAA TCT GCC CTC TGC GCT GGG AAG TGA GCC AGC CAA TCA GTG ACA ATA GAA GAC 1620
TGT CAT ACA AAG AAT CAG TAA AGA CTC TAA CCT TGT TGT TGT TGT TGT TGT TGT TGT 1680
GTC TGA GTC TGG GTC CAT GCT TCA GAA GGG GTA AGC TGA CAT CCA CTG ACT GTA CCT CTC 1740
TGA ACC CAA GGT ACA GAA GAA CAG GAA GCC CCA ATC CAT TTC ATA ATC AAC CCA GAT GCT 1800
GCA GCC CAT ACA GAA TTT GGC CTG AAT GAT TTT TCG AGC ATT AAA TGG AGG CCA AGT 1860
CCA CTC TTT AGA TAT TAA ATG AAT ATT CTT TTG CAA AGG AAAAAAAAAAAAAAAAAAAAAA-3' 1920

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corresponding to the P (pore-forming) region in voltage-gated K^+ channels which constitutes the ion conduction pathway^{45–48}. Significantly, the ROMK1 P region also conserves the tyrosine-glycine couplet (Tyr 144/Gly 145) recently demonstrated to be a requirement for K^+ selectivity in these channels⁴⁹. Single amino-acid substitutions introduced into the P segments of voltage-gated K^+ channels result in the loss of channel function⁵⁰ or alter single channel conductance and ion selectivity^{46,48,51}. In this respect, two amino-acid substitutions, Val 140 and Ile 142, in the ROMK1 H5 region are notable; the corresponding positions in *Shaker* H5 sequences have been shown to alter NH_4^+ permeability⁴⁸ and the Rb^+/K^+ conductance profile⁵¹. Conservation of a P segment by channel proteins with such different structural designs provides additional evidence that this amino-acid segment constitutes the ion permeation pathway in these K^+ -selective channels. Moreover, the finding of an H5 region in ROMK1 suggests a common ancestry with voltage-gated K^+ channels. And yet the degree of similarity shared between ROMK1 and *Shaker* proteins within this region, when compared to that shared by the most distantly related *Shaker* subfamilies (~82%), suggests that the ancestral gene for the ROMK1 protein diverged early in evolution. In keeping with this, the ROMK1 P segment displays an even greater divergence from voltage-gated K^+ channel P regions than do the corresponding segments from either the *Drosophila* Eag⁵² or Slo proteins³¹ (71 and 82% similarity, respectively).

Apart from the P segment, the remainder of the S5–S6 linker in *Shaker* proteins contributes to the external vestibule surrounding the mouth of the channel pore^{44–46,50}. Glycosylation of the ROMK1 protein at Asn 117 is consistent with the predicted extracellular location of the M1–P linker (Fig. 6) and is similar to the potential *N*-linked glycosylation sites in the analogous Shal2, mShal, and Eag S5–P linkers. In addition, amino-acid positions adjacent to the P segment that alter the inhibitory effect of external TEA^+ on *Shaker* channel activity (Asp 431 and Thr 449 in *Shaker* H4) are occupied by Ser 130 and Phe 148 in ROMK1. Using site-directed mutagenesis, others have shown⁴⁶ that a reduction in *Shaker* H4 TEA^+ affinity results from the substitution of asparagine for aspartic acid, a single-charge change, at position 431 through an electrostatic effect. The presence of serine at the corresponding position in ROMK1 would be consistent with a low sensitivity to external TEA^+ . Phenylalanine at position 148, by contrast, would be expected to confer high TEA^+ affinity. In *Shaker* channels, a high-affinity TEA^+ binding site is produced by the substitution of an aromatic residue (Tyr, Phe) at the corresponding position^{46,53,54}. External TEA^+ affinity is substantially reduced, however, if a positively

charged residue occurs in close proximity to the aromatic amino acid⁵⁵. Further, replacement of the aromatic residue by lysine, arginine or histidine is associated with TEA^+ insensitivity^{46,53}. It is likely that the former situation applies to ROMK1 as an arginine (Arg 147) is located adjacent to Phe 148.

Of equal significance to the presence of an H5 region in the ROMK1 protein is the remarkable absence of regions corresponding to the S1, S2, S3 and S4 segments present in all voltage-gated ion channels. The absence of a typical S4 segment, which is thought to form the voltage sensor^{27,56} in these channels, is particularly noteworthy. A region in ROMK1, M0, can be identified as an amino-acid segment of intermediate hydrophobicity (Fig. 4). On the basis of its amphipathic nature and its position relative to the H5 segment of ROMK1 (Fig. 3a), one might expect the M0 region to correspond to an S4 region. Although M0 displays a limited similarity (36%: 24% identity, 12% conserved substitutions) to, for example, the *Shaker* A S4 region, it does not contain the characteristic S4 motif of repeating basic residues at every third position (Fig. 5b). Of seven S4-motif positions for basic residues, every other one is occupied by an uncharged residue in M0, and acidic residues occupy two of the remaining four sites; this results in a net charge of +1 for this segment (compared to +8 in *Shaker* A). Moreover, ROMK1 conserves only the first leucine (Leu 75) of an S4–S5 leucine heptad repeat in voltage-gated ion channels that is involved in *Shaker* voltage-dependent gating⁵⁷. It is reasonable to propose that the relatively shallow voltage-dependence exhibited by ROMK1 gating at membrane potentials in the physiological range might be explained by the deficiency of a functional or archetypal S4 segment.

In view of the activation of ROMK1 channels by Mg-ATP, a search for nucleotide-binding motifs and consensus phosphorylation sites was made. A Walker type-A motif (GX₄GKX₂(I/V)) representing a phosphate-binding loop^{58,59} occurs at position 223 (Fig. 3a) and identifies a single putative ATP-binding site. Interestingly, the motif occurs within a 27-amino-acid segment having 44% identity (67% similarity) to catalytic subdomain I of ERBB3, a member of the epidermal growth factor (EGF) receptor tyrosine kinase subfamily⁶⁰. Subdomain I, which is conserved by protein-serine/threonine kinases and protein-tyrosine kinases, contains the ATP-binding consensus⁶¹ GXGXXG (Fig. 5c). ROMK1 also contains potential protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) phosphorylation sites⁶², several of which occur in close proximity to the Type A motif (Fig. 3a). Clustering of multiple phosphorylation sites in a cytoplasmic domain occurs in the α -subunits of rat brain Na^+ channels⁶³ R_I and R_{II}, in

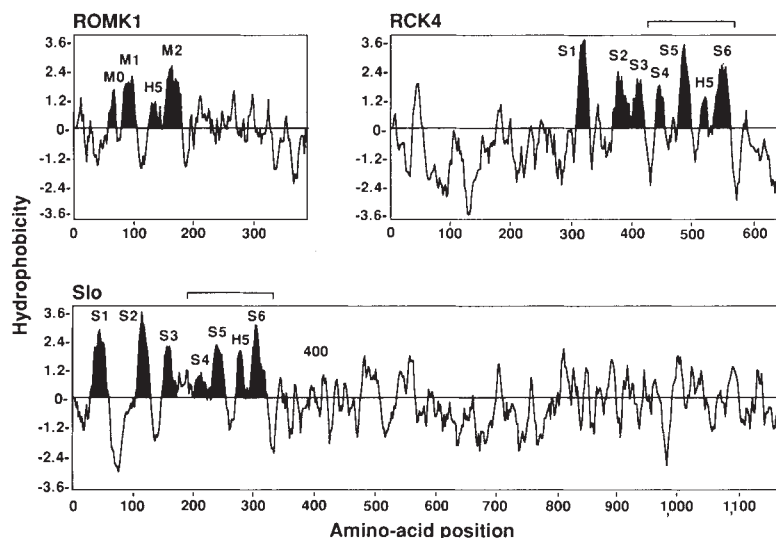


FIG. 4 Comparison of the hydropathy profiles for the deduced polypeptides encoded by ROMK1, RCK4 (ref. 18), and Slo (ref. 31) cDNAs. Hydropathy values were calculated according to ref. 100 (window size of 11 amino acids) and are plotted against amino-acid position. Positive values indicate hydrophobic regions; negative values indicate hydrophilic regions. Shaded hydrophobic peaks correspond to the K^+ channel amino-acid segments indicated. Note that hydrophobic peaks labelled M0–M2 in the ROMK1 plot show some resemblance to peaks labelled S4–S6 (bracketed) in the RCK4 and Slo plots.

ligand-gated ion channel subunits⁶⁴, and in the CFTR protein R-domain⁶⁵. Moreover, a high density of basic amino acids characterizes the region encompassing these sites; all 16 charged residues occurring in the segment 181–234 are positively charged (Fig. 6). No homology was found between the region containing the putative ATP-binding site and the nucleotide-binding domains of ATP-binding-cassette superfamily members⁶⁶, which include the cystic fibrosis transmembrane conductance regulator (CFTR) protein⁶⁵.

A structural model

We propose that the M1 and M2 segments are membrane-spanning and flank the H5 region which is likely to form the channel pore (Fig. 6). In view of the similarity between the H5 regions of ROMK1 and voltage-gated K⁺ channel proteins, the highly K⁺-selective pores formed by these proteins may be structurally similar. Consistent with this possibility is the finding that both channel types conserve the H5 Tyr–Gly motif associated with K⁺ selectivity⁴⁹. Voltage-gated K⁺ channels are homotetramers^{67,68}; the P segments of constituent polypeptides apparently interact to produce an ion conduction pathway lined by eight antiparallel β -strands. In the structural model proposed by Durell and Guy⁶⁹, the *Shaker* channel is composed of 16 outer α -helices (S1, S2, S3, S5) and 8 inner α -helices (S4, S6) surrounding such a pore structure. A channel formed by a tetramer of ROMK1 polypeptides would have to be quite different. A possible model would consist of an ion-conducting P-segment β -barrel supported by a framework of eight (M1, M2) transmembrane helices. We have not excluded the possibility that the ROMK1 channel *in vivo* might include additional subunits or associated proteins.

Whereas the ROMK1 C terminus is probably cytoplasmic, the topology of the amphipathic M0 segment and, subsequently, the location of the N terminus is unclear. Both the absence of an N-terminal hydrophobic signal sequence and the distribution of positively charged residues around the M0 and M1 regions would favour a cytoplasmic N terminus^{70,71}, making it unlikely that M0 spans the membrane. But given the similarity of the M0 sequence to S4 sequences, the M0 segment could conceivably

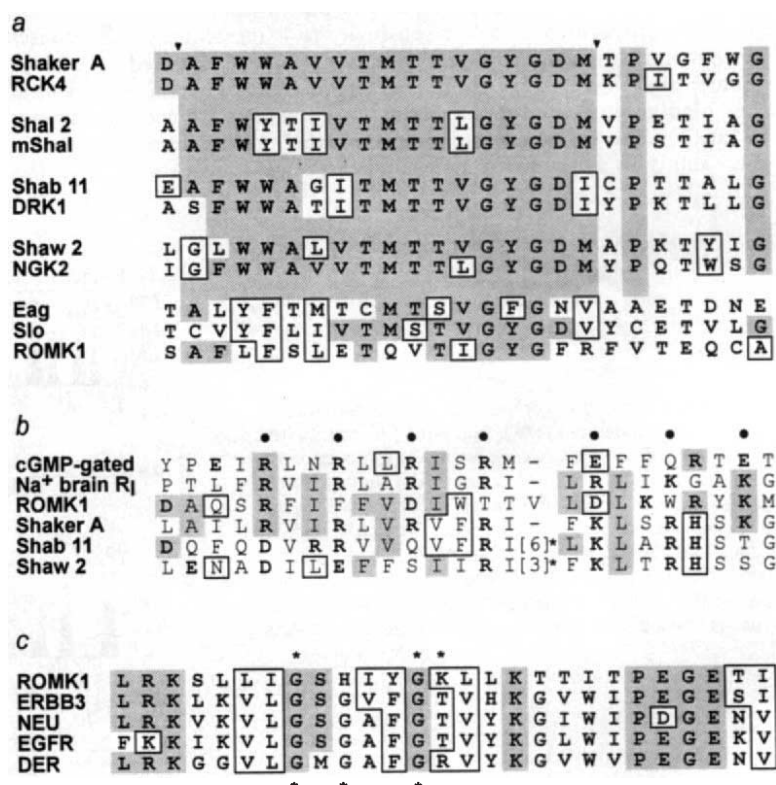
interact with either the M1 and M2 segments or the lipid bilayer in a structural capacity.

The primary structure of ROMK1 also suggests potential mechanisms by which Mg-ATP reactivates channel function following run-down. In Ca²⁺ channels³⁶, K⁺ channels (inward rectifiers^{35,72}, delayed rectifiers^{37,73,74}, K_{ATP} channels^{35,72,75}) and ligand-gated channels (GABA_A receptor)⁷⁶, run-down in excised patches or perfused cells can be partially reversed or prevented by intracellular Mg-ATP through a process apparently requiring ATP hydrolysis. Channel activation by Mg-ATP similarly occurs in Ca²⁺-activated⁷⁷ and muscarinic-activated⁷⁸ K⁺ channels and in K_{Ca,ATP} channels⁷⁹. The principal hypothesis proposed to explain this activation has been the phosphorylation of the channel or of a tightly associated protein by a protein kinase. Studies of squid giant axon delayed rectifiers suggest that the reversal of rundown by Mg-ATP involves phosphorylation-mediated alterations in kinetic and voltage-dependent characteristics of channel activation and inactivation^{73,74}. Modulation of channel activity by the direct phosphorylation of channel polypeptides has been demonstrated in Na⁺, Ca²⁺, K⁺ and ligand-gated channels^{80–83}. The Mg-ATP-dependence of ROMK1 channel activity and the presence of potential PKA and PKC phosphorylation sites suggests the possibility of a similar form of regulation.

A second mechanism for ATP-dependent activation may exist in the single putative ATP-binding site that follows the transmembrane regions of ROMK1 in a manner reminiscent of the nucleotide-binding domains in cyclic nucleotide-gated channels^{29,30} and in CFTR⁶⁵. In segment 181–234, the proximity of the ATP-binding site to a large cluster of basic amino acids and potential phosphorylation sites raises the possibility that these structural features, as an ATP-binding regulatory domain, might participate in an integrated mechanism regulating ROMK1 channel gating.

Such a mechanism combining two distinct Mg-ATP-requiring processes has been proposed for CFTR activation⁸⁴. In the CFTR Cl[−] channel, the highly charged R-domain, which contains multiple PKA and PKC consensus sites, inhibits channel function, possibly through pore occlusion⁸⁵. Phosphorylation of

FIG. 5 a Alignment of the H5 regions from ROMK1, K⁺ channel proteins (*Drosophila*: Shaker A (ref. 3), Shal2, Shab11, Shaw (ref. 11); mammalian: RCK4 (ref. 18), mShal (ref. 20), DRK1 (ref. 15), NGK2 (ref. 16) representing each *Shaker* subfamily, Slo³¹, and Eag⁵² proteins. Using the Shaker A sequence as reference, identical amino acids in the other sequences are shaded; conservative substitutions are boxed. Amino acids in the following groups were considered to be conserved: {M, I, L, V}, {F, Y, W}, {A, G}, {S, T}, {Q, N}, {K, R, H}, {E, D}. The P segment, which forms the ion conduction pathway in voltage-gated K⁺ channels^{46–48}, is delimited by arrowheads. b Alignment of the ROMK1 M0 region with S4 regions of *Shaker* K⁺ channels, cGMP-gated ion channel²⁹, and rat brain Na⁺ channel R₁, repeat IV (ref. 101). Gaps (dashes) have been introduced for maximal alignment. [6]* and [3]* represent amino acid segments MRILRV and MRL, respectively. Charged amino acids are in bold. The seven potential sites for basic residues in *Shaker* S4 sequences are marked (●). c Alignment of catalytic subdomain 1 sequences from members of the EGF receptor tyrosine kinase subfamily (ERBB3, NEU, EGFR, DER)¹⁰² with a 27-amino-acid segment of ROMK1 containing the Walker type-A motif proposed to be involved in nucleotide binding. Putative ATP-binding sites are labelled with asterisks. For b and c, the ROMK1 sequence serves as reference; identical amino acids (shaded) and conservative substitutions (boxed) in the other sequences are shown.



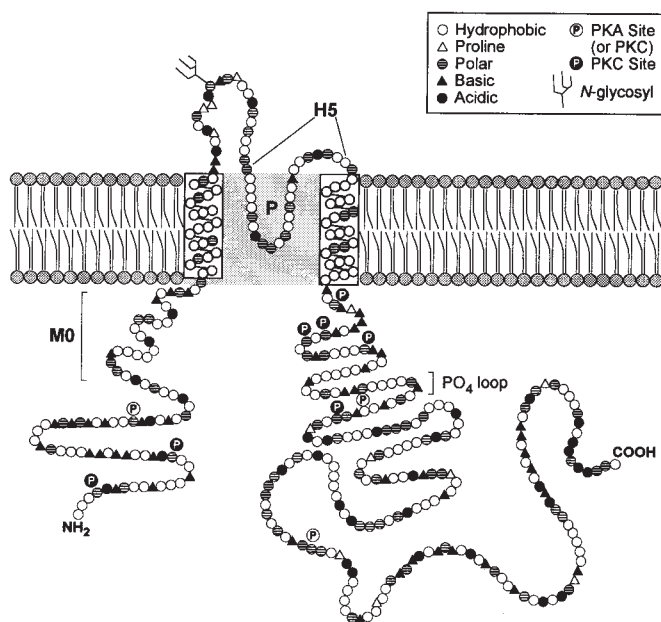


FIG. 6 Structural model for the predicted ROMK1 channel protein. The proposed pore-forming P segment of ROMK1 (P) is located between membrane-spanning segments M1 and M2; the H5 region is indicated. The M0 segment is assumed not to span the membrane, so the N terminus is depicted in a cytoplasmic location. A single putative ATP-binding site identified by the Walker type-A motif (PO₄ loop) is associated with a group of basic amino acids and potential phosphorylation sites and may form a domain (AR domain) directly involved in regulating channel opening. The site for N-linked glycosylation (Asn 117) and potential phosphorylation sites⁶² for protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) (or possibly PKC) are shown. Symbols represent individual amino-acid residues. Amino acids were grouped as follows: {hydrophobic: A, G, M, I, L, V, F, W, C, P}, {uncharged polar: N, Q, S, T, Y}, {acidic: D, E}, {basic: K, R, H}.

this domain, followed by the interaction of Mg-ATP with nucleotide-binding domains, appears to alter the configuration of the R-domain, resulting in channel opening^{84,86,87}.

An analogous model for ROMK1 could be proposed in which the ATP-binding regulatory domain performs both pore-blocking and Mg-ATP-dependent regulatory functions. According to the model, the phosphorylation of this domain and subsequent binding of Mg-ATP counteract an inhibitory interaction of the domain with the channel pore, thereby allowing ion conduction. Cytoplasmic segments containing a high density of basic residues mediate channel inactivation in Na⁺ and Shaker K⁺ channels^{88,89} by obstructing the channel pore through a 'ball-and-chain' mechanism⁹⁰. The positively charged ATP-binding regulatory domain may perform a similar function. A dual regulatory model suggests that there is more than one process underlying the channel inactivation that results from the removal of Mg-ATP: namely dephosphorylation and dissociation of Mg-ATP. These latter processes could be consistent with the run-down, reversible Mg-ATP-dependent activation, and attenuation of this latter effect exhibited by ROMK1 channels in excised patches; similar channel properties have been reported for CFTR channels^{84,91}. Site-directed mutagenesis studies will be needed to elucidate the mechanisms and potential regulatory interactions with other polypeptides that determine ROMK1 gating. □

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LETTERS TO NATURE

High-resolution VLBA imaging of the radio source Sgr A* at the Galactic Centre

K. Y. Lo*, D. C. Backer†, K. I. Kellermann‡, M. Reid§, J. H. Zhao||, W. M. Goss|| & J. M. Moran§

* Astronomy Department, University of Illinois, 1002 West Green Street, Urbana, Illinois 61801, USA

† Astronomy Department, University of California, Berkeley, California 94720, USA

‡ National Radio Astronomy Observatory, Edgemont Road, Charlottesville, Virginia 22903, USA

§ Harvard-Smithsonian Center for Astrophysics, 60 Garden Street, Massachusetts 02138, USA

|| National Radio Astronomy Observatory, Socorro, New Mexico 87801, USA

THE compact non-thermal radio source at the Galactic Centre, known as Sgr A*, may mark the location of a massive black hole^{1,2}. Here we present images of Sgr A* with milliarcsecond resolution obtained by using five telescopes of the partially completed Very Long Baseline Array (VLBA) in conjunction with a few additional telescopes. The image of Sgr A* at a wavelength of 3.6 cm confirms almost exactly the elliptical gaussian model that has been proposed on the basis of previous, more sparse data^{3,4}. The source size at 1.35 cm wavelength is 2.4 ± 0.2 mas, similar to previous results^{3,5}. At both wavelengths, the radio source is smooth, without detectable fine structure. These observations, along with other recent results⁶, support the suggestion⁷ that the radio emission from Sgr A* is strongly scattered by electron-density fluctuations along the line of sight. On the assumption⁸ that the emission is due to a black hole accreting stellar winds from massive stars in the central 0.5 pc, the observations are consistent with a black-hole mass of $\leq 2 \times 10^6 M_{\odot}$.

Since the compact radio source at the Galactic Centre, Sgr A*, was first unambiguously detected in 1974 (ref. 9), there have been many attempts to determine its structure^{3–5,10}, but the lack of radio telescopes with the appropriate combination of short and intermediate spacings meant that the source could not be imaged adequately. The partially completed VLBA now provides the necessary short spacings and has been used in conjunction with other telescopes to image Sgr A* at wavelengths 1.35 and 3.6 cm.

The 1.35-cm observations were made on 28 June 1991, using nine telescopes: five of the 25-m diameter VLBA telescopes (Pie Town, Kitt Peak, Los Alamos, Fort Davis and Owens Valley), the phased Very Large Array (VLA), the NASA Goldstone 70-m telescope, the Haystack 37-m telescope and the NRAO 43-m telescope in Green Bank. The Mk III very-long-baseline interferometry (VLBI) recording terminals were used in mode A (56-MHz recording bandwidth) at the telescopes, except for the VLBA telescopes where the VLBA recorders were used in a compatible mode (50-MHz recording bandwidth). Data recorded from the Owens Valley VLBA telescope and the Goldstone 70-m telescope could not be used, because of technical difficulties at the time of observations. At short wavelengths such as 1.35 cm, the antenna gain changes, and atmospheric attenuation

becomes significant and difficult to calibrate at the low elevations at which Sgr A* is observed (from the Northern Hemisphere). To overcome these difficulties which limit the accuracy of the determination of the visibility amplitudes, we observed an H₂O maser source in Sgr B2 at the end of each scan of Sgr A*. This maser source, being ~ 0.7 degree away from Sgr A*, allows reliable calibration of the visibilities of Sgr A*. We also observed the unresolved source 1730–130 (NRAO 530) as a check on the calibration. Using the VLA data, we determined the total flux density of Sgr A* to be 0.98 ± 0.05 Jy during the observations.

The 3.6-cm observations were made on 25 November 1991 using seven telescopes: the same five VLBA telescopes, the phased VLA and the NASA Goldstone 70-m telescope. The VLBI recordings were made with a bandwidth of 28 MHz. Observations alternated between Sgr A* and the neighbouring calibrator source 1730–130 (NRAO 530). The measurements at the VLA during the observations of Sgr A* were used to determine the flux density of Sgr A* as 0.79 ± 0.05 Jy.

The data were correlated at the Haystack Observatory on the Mk IIIA correlator¹¹ and the correlation coefficients were then edited, calibrated and analysed using the Caltech VLBI package.

Sgr A* was detected on the short and intermediate baselines, whereas the calibrator source NRAO 530 was detected on all the baselines. The measured visibility amplitudes were well fitted by the amplitudes of an elliptical gaussian brightness distribution. The major axis angular diameter (FWHM) is 17.5 ± 0.5 mas, with an axial ratio of 0.49 ± 0.06 , and the position angle (PA) of the major axis at $87^\circ \pm 5^\circ$.

Using the standard procedure of hybrid mapping¹², we have also derived the image shown in Fig. 1. The source is well

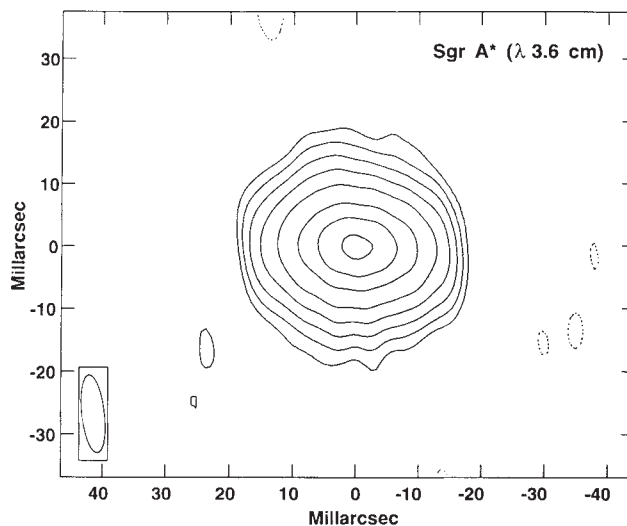


FIG. 1 'Hybrid' image of Sgr A* at wavelength 3.6 cm. The beam is $12.4 \text{ mas} \times 6.6 \text{ mas}$ (PA = 6.6°). The contour levels are $-2, 2, 4, 8, 16, 32, 64, 96$ and 128 mJy per beam . A typical r.m.s. noise in this image is $0.45 \text{ mJy per beam}$. The origin of the image is at RA(1950) = $17 \text{ h } 42 \text{ min } 29.34 \text{ s}$, Dec(1950) = $-28^\circ 58' 18.6''$.

resolved by the synthesized beam of $12.4 \text{ mas} \times 3.6 \text{ mas}$ (major axis PA = 6.6°). An elliptical gaussian model fitted to the deconvolved image has the following parameters: major axis (full width half maximum, FWHM) = $17.5 \pm 0.5 \text{ mas}$, minor axis (FWHM) = $8.5 \pm 1.0 \text{ mas}$, major axis PA = $87^\circ \pm 3^\circ$, essentially identical to a model fitting the visibility amplitudes.

There is no evidence of any fine structure at angular scales smaller than the gaussian source size in either the visibility amplitudes or the hybrid image. Further evidence that the source structure is smooth and symmetric can be seen in the closure phase data. Whenever the closure phase can be measured, it is consistent with zero, implying an absence of any asymmetric small-scale structure.

The 1.35-cm visibility amplitudes are well fitted by a circular gaussian model with a diameter of $2.4 \pm 0.2 \text{ mas}$ (Fig. 2). If we fix the axial ratio at 0.5 and the PA of the major axis at 87° , the same data can also be modelled by an elliptical gaussian brightness distribution, with a major axis diameter of $2.6 \pm 0.2 \text{ mas}$. The currently limited north-south (u, v) coverage of the partial VLBA does not provide sufficient constraint on the ellipticity of Sgr A* at 1.35 cm. But an independent experiment at 1.35 cm (using the same telescopes but without the special calibration using the H₂O maser source in Sgr B2) reported an elliptical gaussian model for Sgr A*: major axis (FWHM) = $2.6 \pm 0.2 \text{ mas}$, PA = $75^\circ \pm 10^\circ$ and an axial ratio = 0.5 ± 0.2 (ref. 13).

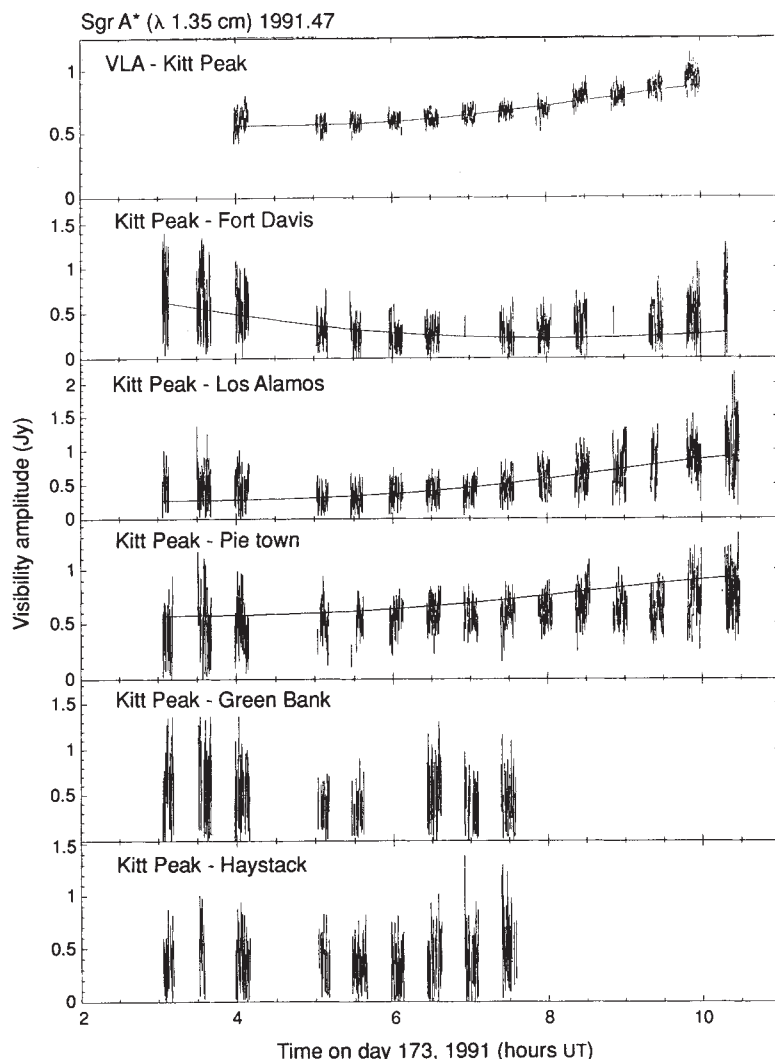
These results, based on the more extensive current data using the partially completed VLBA, confirm the apparent source

structure of Sgr A* previously determined from much sparser data⁶⁻⁸. Given the definitive determination of the elliptical gaussian shape of Sgr A* at 3.6 cm, comparison to images at other wavelengths is now required to examine the wavelength (λ) dependence of the source size as well as source shape. The λ^2 dependence of size scale noted before may be too simplified a description. Comparison of the source sizes measured at $\lambda = 1.35 \text{ cm}$ over 8 years can, however, now be used to set an upper limit of 0.04 mas yr^{-1} to any change in the source size (corresponding to a radial expansion velocity of $\sim 0.8 \text{ km s}^{-1}$ at 8.5 kpc)³. If we assume that the observed size is dominated by interstellar scattering, then the upper limit can be two times larger.

It has been suggested⁷ that the observed source size of Sgr A* is affected by scattering of interstellar electrons, but the large observed size of Sgr A* requires rather extreme conditions¹⁴. The wavelength dependence of the observed source size of Sgr A* could also be due to the synchrotron and free-free opacity of the source¹⁵, but *ad hoc* assumptions are needed to reproduce the λ^2 dependence. Recent results from OH maser source-size measurements indicate a $\sim 30'$ region of pronounced scattering centred on Sgr A* (ref. 6), strengthening the interpretation of interstellar scattering.

If the observed source structure of Sgr A* at 3.6 cm is due to interstellar scattering, the measured visibilities should pose some constraints on the parameters characterizing the structure of the interstellar electrons. The expected relation is $P_{\delta n_e}(q) = C_n^2 q^{-\alpha}$,

FIG. 2 Visibility amplitudes of Sgr A* at wavelength 1.35 cm plotted against time (hr) for six baselines all involving Kitt Peak. The solid curves are the predicted amplitudes for a circular gaussian model source with a FWHM diameter of 2.5 mas. There are no model curves plotted on the baselines from Kitt Peak to Green Bank and Haystack because the predicted amplitudes are vanishingly small. The amplitudes are consistent with pure noise, given the short integration time used to minimize decorrelation of the visibility due to the atmosphere.



where $P_{\delta n_e}$ is the power spectrum of the electron density fluctuations as a function of the wavenumber, $q = 2\pi/b$ (b is the scale size of the fluctuations), in the range $2\pi/b_1$ to $2\pi/b_0$, where b_0 and b_1 are the inner and outer scales, respectively⁶. The VLBI image is derived from observations over about 7 hours, a time-scale longer than that for diffractive scattering, t_{diff} , but short compared with the timescale for refractive scattering, t_{ref} . The image obtained by VLBI is thus an 'average' image, as defined by Goodman and Narayan¹⁶ who analysed the dependence of the measured visibility on projected baseline length b (and therefore scale size in the scattering medium) for various parameters of the power spectrum $P_{\delta n_e}$. The profile of $V(b)$, the normalized visibility V against projected baseline length b , is sensitive to the index of the power spectrum under certain conditions^{17,18}.

We have fitted $\ln(1/V(b))$ to a power-law dependence on b (corrected for ellipticity of the image) with data selected for sufficient signal to noise ratio, yielding a power law index of 2.05 ± 0.15 , where the errors are the bounds of fits with different data selections¹⁹. This value can be taken to imply that the power-law index, α , of $P_{\delta n_e}$ is 4.05, similar to that determined previously²⁰ but higher than that found for other objects^{17,19} and higher than the value of a Kolmogorov power spectrum ($\alpha = 11/3$). On the other hand, it could be consistent with a Kolmogorov power spectrum with an inner scale of turbulence b_0 that is larger than the maximum baseline on which Sgr A* is detected, ~ 450 km (ref. 19). The λ -dependence of the measured major-axis sizes ($\lambda^{1.94 \pm 0.03}$) is consistent with either possibility.

One might expect some deviation of $V(b)$ from a smooth bivariate gaussian form with our integration time, but such effects should be small relative to both the gain errors in our data and the limits we can set on $V(b)$ on the longer baselines ($V < 0.05$). For example, deviations at the level of $V \sim 0.01$, presumably due to this effect, have been detected in another radio source²¹. On our most sensitive closure-phase triangle (Goldstone, VLA and Pie Town), there is no evidence for a detectable source on the long baseline Goldstone-VLA at the level of $V(b) = 0.01$.

The anisotropy of the image (in terms of both axial ratio and position angle) is unchanged over a timescale of ~ 7.5 years, the interval between the 3.6-cm observations reported here (November 1991) and the first detection of elongation of the source (May 1983)¹⁰, which is longer than t_{ref} . This 'permanent' anisotropy suggests an anisotropy in the spectrum of density fluctuations. In general, the image will be stretched out perpendicular to the direction along which the projected density blobs are elongated¹⁶, which could be due to strong and smooth magnetic field threading the turbulent region or to anisotropic velocity fields arising from stellar winds or supernovae¹⁷.

The unique properties of the radio source Sgr A* in the Galaxy cannot be explained by any known stellar radio source^{2,3}. It may be best explained by a massive black-hole model²², but its mass is not known. The absence of proper motion of Sgr A* is consistent with its being massive²³. Direct determination of the mass distribution at the centre is clearly necessary. The best kinematic evidence for the presence of a large point mass at the Galactic Centre is from modelling the high-speed ionized gas within the central few parsecs²⁴. Similar evidence from stellar velocity dispersion is also suggestive, although the current uncertainties of the core radius and the mass-to-light ratio of the central star cluster constrain the mass model²⁵⁻²⁷. The Sgr A* radio source has been modelled as a massive black hole accreting the fast wind generated by the sources in IRS16 (ref. 8). If one uses the predicted intrinsic source size of this model²⁸, the 1.35-cm measurement of the source size can be interpreted to exclude a black-hole mass of $> 2 \times 10^6$ solar masses ($M_\odot$). Given the many assumptions involved in the modelling, such limits on the mass are uncertain. The quest for the intrinsic source structure of Sgr A* requires further VLBA imaging at shorter

wavelengths, where scattering by interstellar electrons (proportional to λ^2) decreases. Together with high-resolution infrared observations of the stars and gas in the central parsec, such observations will help to determine the true nature of the centre of the Galaxy. □

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Microsecond-resolved XAFS of the triplet excited state of $\text{Pt}_2(\text{P}_2\text{O}_5\text{H}_2)_4^{4-}$

Daniel J. Thiel*, Pēteris Liviņš†, Edward A. Stern† & Aaron Lewis*‡

* Department of Applied Physics, Cornell University, Ithaca, New York 14853, USA

† Department of Physics, FM-15, University of Washington, Seattle, Washington 98195, USA

‡ Division of Applied Physics, The Hebrew University of Jerusalem, Jerusalem, Israel

LITTLE is known about the excited-state structures of most inorganic compounds. Time-resolved resonance Raman and time-resolved infrared spectroscopies can provide only indirect structural information for short-lived excited species in solution at room temperature. Time-resolved X-ray diffraction¹ has the potential to give more direct information, but no excited-state structures have yet been reported; picosecond gas-phase electron diffraction has been proposed recently², but not yet demonstrated. Here we report a technique that combines the X-ray absorption fine-structure (XAFS) method³ with rapid-flow laser spectroscopy⁴ to measure structural changes in a solution-phase excited-state transition-metal complex with microsecond resolution. We find that the triplet excited state of $\text{Pt}_2(\text{P}_2\text{O}_5\text{H}_2)_4^{4-}$, with a lifetime of about 4 μs , undergoes a contraction in the Pt-Pt distance of 0.52 ± 0.13 Å relative to the ground state. We anticipate that time-resolved XAFS will have broad applications in chemistry and biology.

Tetrakis(pyrophosphito)diplatinate(II), or $\text{Pt}_2(\text{P}_2\text{O}_5\text{H}_2)_4^{4-}$ consists of two platinum centres with four bridging pyrophosphite ligands as shown in Fig. 1. The structure, spectroscopy, reactivity and photochemistry of this molecule have been extensively studied^{5,6}. Several of its characteristics make it a good subject for our microsecond XAFS technique. First, its triplet excited state, denoted $(\text{P}_2\text{O}_5\text{H}_2)_4^{4-*}$, has a fairly long room-temperature lifetime of 9 μs . Second, this state can be significantly populated by laser irradiation because the ground-state absorption is high ($\epsilon = 34,500 \text{ M}^{-1} \text{ cm}^{-1}$ at 368 nm) and the quantum yield of formation is large (0.5). Third, a large contraction ($\sim 0.2 \text{ \AA}$) of the Pt-Pt separation on excitation has been inferred from vibrational analyses^{7,8}.

Structural studies of the triplet state have previously been limited to indirect methods of investigating the Pt-Pt separation⁷⁻¹⁰. With time-resolved XAFS to probe $\text{Pt}_2(\text{P}_2\text{O}_5\text{H}_2)_4^{4-*}$, we can directly determine the alterations in the positions of the phosphorus atoms on excitation. Our kinetic XAFS technique involves a circulation system which allows rapid flow of the solution under investigation, a laser with appropriate optics to excite and monitor the molecule, and a synchrotron radiation source with the requisite instrumentation for X-ray absorption spectroscopy. The solution is passed through a nozzle to form a free-standing jet, similar to that of a dye laser. The sample is excited by focusing the laser beam on the jet, and the X-ray beam is subsequently directed at the jet downstream of the laser focus (Fig. 2). If the pump and probe regions do not overlap, the time resolution is limited by time τ for which the flowing molecules reside in the laser focus and the X-ray beam. This is given by $\tau = h/v$ where $h = h_1 + h_2$ is the combined height of the two beams (see Fig. 2).

The experiment was done under partially deoxygenated conditions. This was to reduce the O_2 quenching¹¹ of $\text{Pt}_2(\text{P}_2\text{O}_5\text{H}_2)_4^{4-*}$ while exploiting the effect of O_2 in regenerating $\text{Pt}_2(\text{P}_2\text{O}_5\text{H}_2)_4^{4-}$ from the unwanted dihydride photoproduct $\text{Pt}_2(\text{P}_2\text{O}_5\text{H}_2)_4\text{H}_2^{4-}$ formed when the excited state reacts with the glycerol¹². The resulting triplet lifetime was 4 μs , obtained independently by both monitoring the decay of the phosphorescence upon excitation with a 10-ns 355-nm pulse from a Nd:YAG laser and by monitoring the transient absorption using the 363-nm continuous laser as the pump/probe source. By translating the pump beam, we found the configuration that gave the maximum excitation within the probe region. Theoretically, this corresponded to a d value of 12 μm or a time delay of 0.8 μs . This value is small compared with the 4- μs triplet lifetime; we note, however, that the resolution based on the

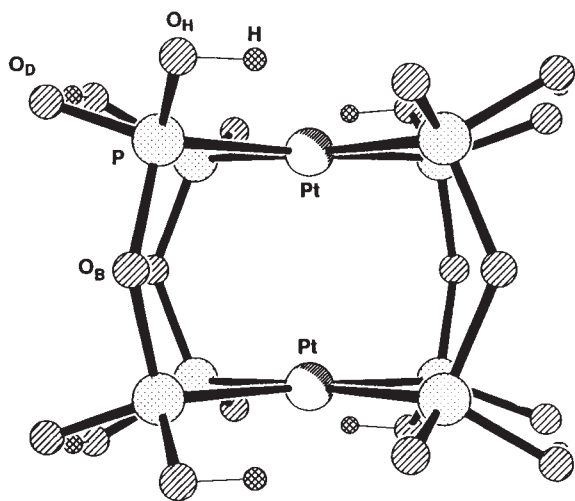


FIG. 1 Perspective drawing of $\text{Pt}_2(\text{P}_2\text{O}_5\text{H}_2)_4^{4-}$ where data from ref. 17 was used. Subscript B denotes a bridging oxygen, H denotes an oxygen which has a hydrogen attached, and D denotes an oxygen which is double-bonded to the phosphorus.

beam heights used was $\sim 1.5 \mu\text{s}$. With this configuration, the optical probe showed that 18% of the molecules within the probed region were in the triplet excited state. The rate of formation of the hydride photoproduct¹³, which subsequently gives rise to the dihydride¹², was slow compared with our timescale.

We chose to study the Pt L_{III} absorption edge. As the sample was dilute ($\Delta\mu x = 10^{-3}$ at the Pt L_{III} edge where μx is the X-ray optical density), we had to use the fluorescence to measure the X-ray absorption. The fluorescence X-rays were collected with a Si(Li) solid-state detector with a typical count rate of 12,000 counts s^{-1} , 20% of which was the fluorescence signal. Data were collected over 24 hours (120 scans). The integrity of the sample was periodically checked using an ultraviolet-visible spectrophotometer; over the collection period it lost $\sim 7\%$ of its absorption at 363 nm. This weakened absorption was not accompanied by any strengthened absorption peaks but only by an increase in light scattering. The spectra were not adversely affected, as the excited-state data showed no significant changes throughout the measurement.

Following standard XAFS analysis, the X-ray absorption fine structure, $\chi(k)$, is obtained using the relation

$$\chi(k) = \frac{\mu(k) - \mu_0(k)}{\mu_0(E_0)} \quad (1)$$

where μ is the measured X-ray absorption coefficient above the edge energy, μ_0 is the smoothly varying background function, and E_0 is the edge energy. Details of the analysis will be given

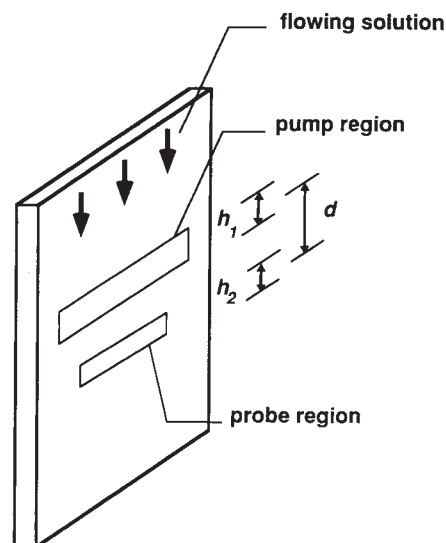


FIG. 2 Schematic view of the interaction region of the jet. Standard procedures²⁰ were followed in preparing the tetrabutylammonium (TBA) salt, $(\text{TBA})_4[\text{Pt}_2(\text{P}_2\text{O}_5\text{H}_2)_4]$. To obtain a sample concentration of 0.7 mM, 300 mg of $(\text{TBA})_4[\text{Pt}_2(\text{P}_2\text{O}_5\text{H}_2)_4]$ was dissolved in $\sim 200 \text{ ml}$ of aqueous solution containing 76% glycerol by weight. With a solution temperature of 16°C and a pressure of 310 kPa, the flow velocity was $\sim 15 \text{ m s}^{-1}$. Two crossed cylindrical lenses defined the pump region by focusing 0.75 W of continuous-wave 363-nm light from an argon ion laser to a line $10 \mu\text{m}$ high (h_1) and $930 \mu\text{m}$ wide on the jet 3 mm below the nozzle. Here the measured optical density of the jet was 0.85 at 363 nm, corresponding to a jet thickness of $420 \mu\text{m}$. As the flow velocity was $\sim 15 \text{ m s}^{-1}$, the molecules were effectively excited by a 0.67- μs pulse, which should excite a significant portion of them to the triplet state. The probe region, a distance d away, was given by an X-ray beam $15 \mu\text{m}$ high (h_2) and $250 \mu\text{m}$ wide at the jet with an overlapping low-power beam of 363-nm light (0.4 mW, $14 \mu\text{m}$ high, $440 \mu\text{m}$ wide) split from the pump beam. All beam sizes were determined by translating a knife-edge through the beam and noting when this reduced the beam power from 90% to 10% of the full power. By varying d , the displacement between the laser beam and the X-ray beam, one can monitor the kinetics of the decay of the excited state. This parameter d provides the time delay Δt given as $\Delta t = d/v$ where v is the velocity of the flowing sample. The X-ray source was the A-2 wiggler at the Cornell High-Energy Synchrotron Source.

elsewhere. Briefly, we used an improved background-finding procedure¹⁴ to extract the functions $\chi_m(k)$ and $\chi_g(k)$ from the excited-state and ground-state $\text{Pt}_2(\text{P}_2\text{O}_5\text{H}_2)_4^{4-}$ data, respectively (Fig. 3a). Since the excitation was only partial, $\chi_m(k)$ is a mixture of the ground-state XAFS $\chi_g(k)$ and the pure excited-state XAFS $\chi_e(k)$. Figure 3b shows the difference spectrum $\Delta\chi(k) = \chi_m(k) - \chi_g(k)$, which may also be expressed as $\Delta\chi(k) = 0.18(\chi_e(k) - \chi_g(k))$ due to the extent of the excitation being 18%. Changes in χ due to the laser excitation can be distinguished out to $8 \text{ \AA}^{-1}$, at which point statistical noise starts to dominate.

Qualitative information about the change in molecular structure in going from the ground to the triplet state can be immediately inferred from Fig. 3b. The phase shift between $\Delta\chi$ and χ_g indicates that there has been a change in separation of the Pt atom and at least one of its near neighbours. If the difference in structure between the two states was due to a change in coordination number around the Pt atom, $\Delta\chi$ and χ_g would be in phase. In the simplest case of only one near-neighbour shell changing position, a change of $\sim 0.4 \text{ \AA}$ may be inferred from the amplitude of $\Delta\chi$.

To obtain quantitative information about $\text{Pt}_2(\text{P}_2\text{O}_5\text{H}_2)_4^{4-}$, we fitted $\Delta\chi$ using the computer code FEFF¹⁵, which calculates the XAFS amplitudes and phases. The accuracy of FEFF for the present problem was verified by fitting χ_g and incorporating into the calculation the known structure of the ground state as determined from Raman scattering¹⁰, infrared absorption¹⁶ and X-ray diffraction¹⁷.

For the data between 3 and $8 \text{ \AA}^{-1}$, we Fourier-transformed $k\Delta\chi(k)$ to r -space and fitted a FEFF calculation to the resulting real and imaginary parts. We determined the values of three fitting parameters: $\Delta r_1 = -0.047 \pm 0.011 \text{ \AA}$, $\Delta r_2 = -0.285 \pm 0.065 \text{ \AA}$, and $\Delta r_3 = -0.06 \pm 0.05 \text{ \AA}$, corresponding to changes in radial separation between the absorbing Pt atom and the near P, the far P, and the bridging O, respectively. Statistical noise precluded the determination of any other XAFS parameters. The fit is shown in Fig. 4. The uncertainties are verified in Fig. 4b where we plot the functions obtained when

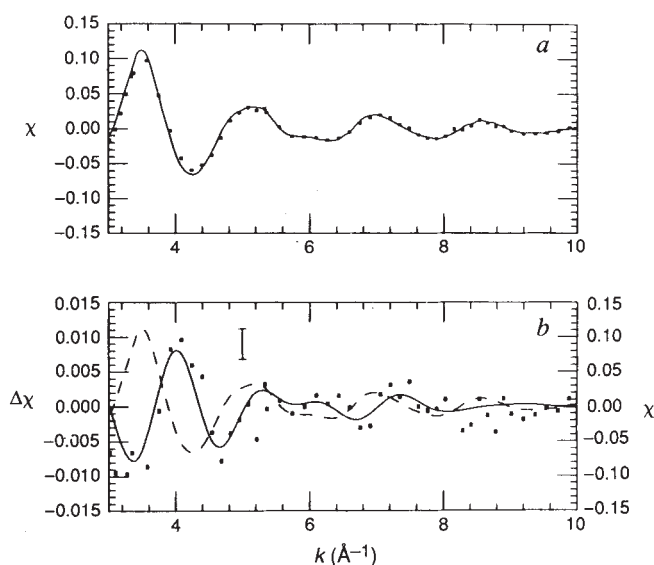


FIG. 3 The XAFS data shown in k -space. *a*, Fine structure of the ground state, $\chi_g(k)$ (solid line), and that of the mixture-state, $\chi_m(k)$, (dots). The uncertainty is as small as the plotting symbols. Significant differences in both phase and amplitude are seen out to $8 \text{ \AA}^{-1}$. *b*, Difference spectrum, $\Delta\chi(k) = \chi_m(k) - \chi_g(k)$, showing the differences in phase and amplitude (dotted line). The error bar indicates the uncertainty. For comparison, χ_g (dashed line) is overlaid. Note the phase difference between it and $\Delta\chi(k)$. Also plotted is the filtered back-transform of our r -space fit (solid line) showing its similarity to $\Delta\chi(k)$.

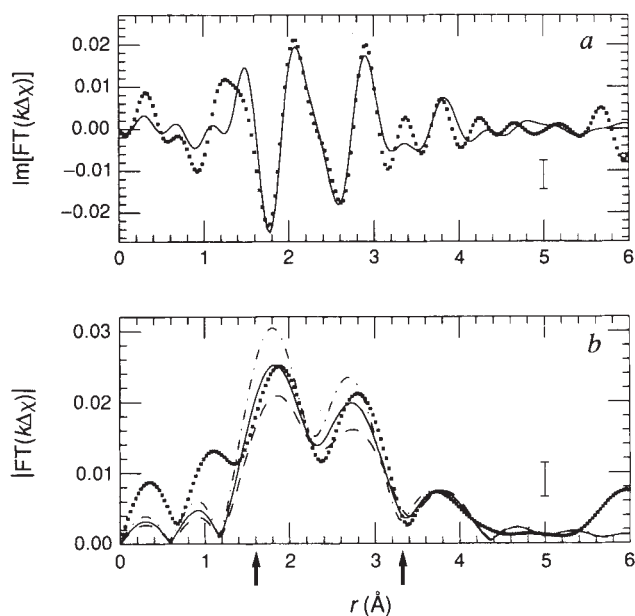


FIG. 4 Comparisons of the data (dots) and the fit (solid line) in r -space. The uncertainty in the data is given by the error bars. *a*, Imaginary component of the Fourier transform of $k\Delta\chi(k)$, with the fit. *b*, Magnitude of the transform. In *b* we also show the fits (dash, dash-dot) where all three fitting parameters are increased and decreased by a value consistent with the r.m.s. uncertainty of the raw data. The arrows indicate the fitting range.

the best-fit values of the fitting parameters Δr_1 , Δr_2 and Δr_3 are increased and decreased by a value consistent with the r.m.s. uncertainty of the raw data. As a final check we back-transformed a filtered portion ($1.6\text{--}3.3 \text{ \AA}$) of the r -space result to generate the function $\Delta\chi(k)$. This agreed, within uncertainties, with the experimentally obtained function shown in Fig. 3b.

Because sensitivity to the Pt-Pt distance is limited by the noise, we use the previously reported excited-state Pt-Pt distance of $2.75 \text{ \AA}$ (ref. 7). Assuming that the phosphorus atoms remain planar in the triplet state, by simple geometry the values of Δr_1 and Δr_2 translate into an inward movement of the P planes along the Pt-Pt axis by $0.52 \pm 0.13 \text{ \AA}$, with a smaller contraction of $0.047 \pm 0.011 \text{ \AA}$ of the Pt-P bond within the planes on excitation. The contraction between the planes is roughly twice that previously found^{7,8} for the Pt-Pt bond. This indicates that the excitation involves molecular orbitals with appreciable ligand character rather than simply Pt atomic orbitals, consistent with a quantum mechanical calculation¹⁸.

This redistribution of charge apparently strengthens not only the P-O_B bonds but also the P-Pt bonds, causing both to contract. The P-Pt contraction is analogous to the predicted Ni-C contraction¹⁹ of the square-planar complex $\text{Ni}(\text{CN})_4^{2-}$.

We have determined relative rather than absolute atomic distances; that is, we have extracted changes in atomic positions within the triplet relative to the ground-state structure. Such an analysis easily yields the $0.01\text{-\AA}$ precision reported here; however, the uncertainties in our parameters are large, typically 20%. We expect that other interesting results will emerge from this technique, especially when applied to biological molecules. □

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Storage of light energy by photoelectron transfer across a sensitized zeolite–solution interface

Marlon Borja & Prabir K. Dutta*

Department of Chemistry, The Ohio State University,
120 West 18th Avenue, Columbus, Ohio 43210, USA

* To whom correspondence should be addressed

A COMMON strategy for storage of solar energy involves the photoexcitation of a donor molecule D followed by electron transfer to an acceptor A. To exploit this strategy in a practical context, a way must be found to impede the back-reaction in which the electron is transferred from A to D (ref. 1). Previous attempts to achieve long-lived charge separation have involved the use of D–A combinations held in well defined geometries by spacer groups^{2,3} or immobilized on supports such as porous media^{4–12}. Immobilization of the redox species poses problems, however, for their subsequent separation in order to reclaim the stored energy. Here we report a system that achieves efficient D–A electron transfer, a slow back-reaction and easy separation of the products. We trap the photosensitizer donor, trisbipyridine ruthenium(II), in the supercages of zeolite Y, and use as the acceptor a neutral, zwitterionic viologen in the surrounding solution. Electron transfer from the ruthenium centre to the viologen is mediated by *N,N'*-tetramethylene-2,2'-bipyridinium ions loaded into the zeolite by ion exchange. Isolation of the donor within the zeolite from the acceptor in the solution outside makes the photochemically generated products easily accessible. Practical utilization of this trimolecular redox assembly will, however, require improvement of the quantum yield.

In previous attempts to develop an artificial photosynthetic system, Willner *et al.*¹⁰ used photoexcited trisbipyridine ruthenium(II) ($\text{Ru}(\text{bpy})_3^{2+}$) and propylviologen sulphonate (PVS) as the donor and acceptor respectively; the reduction potentials are, respectively, -0.87 V (ref. 13) and -0.41 V (refs 14, 15) relative to the normalized hydrogen electrode (NHE). The thermal back-reaction was slowed down in the presence of colloidal silica particles, as the PVS radical was electrostatically repelled by the negatively charged surface groups on silica¹⁰. Krueger *et al.*¹¹ showed that for a covalently held $\text{Ru}(\text{bpy})_3^{2+}$ –viologen complex, longer-lived charge separation could be attained by transferring the electron from the covalently bound viologen to another viologen in zeolite L cavities. Slama-Schwark *et al.*¹² obtained charge separation by using a mobile electron relay to transfer electron from photoexcited pyrene to viologen, both held rigidly in a sol-gel glass.

We have incorporated aspects of these previous studies in a host system of zeolite Y, (Na–Y). The framework of this zeolite is made up of tetrahedrally connected sodalite cages, resulting in large supercages (Fig. 1a). The unit cell composition of this aluminosilicate crystal is $\text{Na}_{53}[(\text{AlO}_2)_{53}(\text{SiO}_2)_{139}] \cdot 250 \text{H}_2\text{O}$, and it is cubic with a dimension of ~ 25 Å (ref. 16). Thus a typical particle of $1 \mu\text{m}$ dimension contain hundreds of these interconnecting supercages, producing a continuous intracrystalline space. The sensitizer molecule $\text{Ru}(\text{bpy})_3^{2+}$ is synthesized inside the supercages and the 12 -Å dimension of this molecule makes for a secure fit inside the 13 -Å supercage^{17,18}. Once synthesized, the $\text{Ru}(\text{bpy})_3^{2+}$ is trapped inside the supercage leaving little room for other species, as the 7 -Å window will not allow it to escape. The windows are large enough however, for photoexcited $\text{Ru}(\text{bpy})_3^{2+}$ to interact with molecules in the neighbouring cages¹⁹. As long as the loading level of $\text{Ru}(\text{bpy})_3^{2+}$ is less than 1 for every 10 supercages and the zeolite remains hydrated, the intrazeolitic environment resembles an aqueous solution¹⁸.

Photolysis (420–680 nm, 200 mW) of the surface of a 20-mg pellet of $\text{Ru}(\text{bpy})_3^{2+}$ –NaY (concentration of $\text{Ru}(\text{bpy})_3^{2+}$,

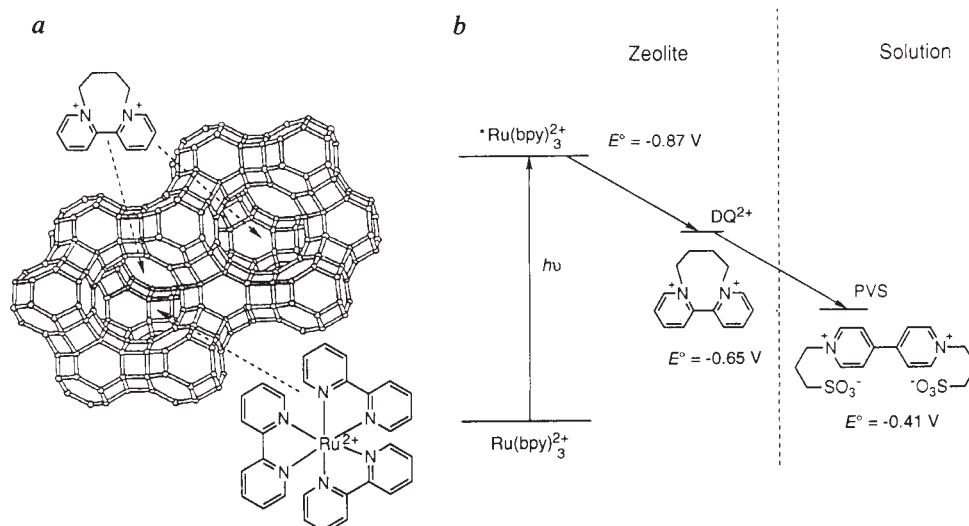


FIG. 1 a, A schematic representation of the entrapped $\text{Ru}(\text{bpy})_3^{2+}$ in zeolite Y, with DQ^{2+} in neighbouring supercages (viewed through the 7 Å windows). b, Vectorial interfacial photoelectron transfer from the zeolite to solution.

$50 \mu\text{mol g}^{-1}$, 1 molecule per 10 supercages) suspended in a 0.01 M solution of propylviologen sulphate (PVS) leads to the appearance of the viologen radical in solution in an hour. (For optimum yields of the radical, it is necessary to deoxygenate the samples thoroughly and keep them in an anaerobic atmosphere during photolysis. This was done by degassing the $\text{Ru}(\text{bpy})_3^{2+}$ -zeolite Y at 10^{-4} Torr for 12 hours. Freeze-pump cycles were done on the 0.01 M PVS solution three times and mixed with the zeolite samples in an anaerobic glove box.) The growth of the radical (measured by the 395 nm band, molar absorptivity $37,000 \text{ l mol}^{-1} \text{ cm}^{-1}$) as a function of photolysis time is shown in Fig. 2a. Comparison of the initial PVS content in solution with that found after interaction with the zeolite showed no perceptible uptake of PVS from the solution into the zeolite. This is not surprising, as there is no driving force for ion-exchange into the zeolite because of the neutrality of the PVS molecule. The photochemical charge transfer from the Ru centre to the viologen must be occurring at the zeolite-solution interface. The negatively charged viologen radical formed on photoexcitation is repelled by the anionic zeolite framework, thereby promoting charge separation. In contrast to the previous work on silica gel¹⁰, in which the back electron transfer was slowed down, we are actually observing charge separation, presumably due to the complete encapsulation of $\text{Ru}(\text{bpy})_3^{2+}$ and the higher charge density arising from the aluminate units on the zeolite framework. For colloidal SiO_2 in the pH range of 7–8, the charge density expressed as OH^- ions nm^{-2} is 0.1–0.25, whereas, with aluminosilicates, the charge density can readily

exceed 2 negative charges per nm^2 (ref. 20). Although this long-lived charge separation is interesting in its own right, the yield of photoinduced redox species is low because only the entrapped $\text{Ru}(\text{bpy})_3^{2+}$ with direct access to the solution viologen species takes part in the photochemical process.

To allow more of the $\text{Ru}(\text{bpy})_3^{2+}$ in the zeolite particles to react, the $\text{Ru}(\text{bpy})_3^{2+}$ -Na-Y was ion-exchanged completely with N,N' -tetramethylene-2,2'-bipyridinium (DQ^{2+}). The loading of DQ^{2+} was found to be 1.1 molecule per supercage, excluding those cages that contain $\text{Ru}(\text{bpy})_3^{2+}$. Thus, all of the $\text{Ru}(\text{bpy})_3^{2+}$ are surrounded by DQ^{2+} in neighbouring supercages. We chose DQ^{2+} on the grounds that its reduction potential of -0.65 V (relative to NHE) is lower than that of the PVS of -0.41 V (relative to NHE) in solution^{14,15}. Also, as PVS is a neutral compound it does not replace the DQ^{2+} in the zeolite.

The $\text{Ru}(\text{bpy})_3^{2+}$ - DQ^{2+} -Y was photolysed in a 0.01 M PVS solution under conditions identical to that of $\text{Ru}(\text{bpy})_3^{2+}$ -Na-Y. The electronic spectrum (Fig. 2b) shows the growth of the PVS $^{\cdot-}$ radical in solution at 60-minute intervals, and Fig. 2a compares this growth with that of the $\text{Ru}(\text{bpy})_3^{2+}$ -Na-Y system. The rate of radical formation is an order of magnitude higher than the sample without DQ^{2+} . Thus, vectorial electron transfer is occurring from the photoexcited $\text{Ru}(\text{bpy})_3^{2+}$ to DQ^{2+} in the zeolite, followed by electron transfer across the zeolite-solution interface to PVS, as shown schematically in Fig. 1b.

To confirm the proposed transfer mechanism, we examined a system in which the intrazeolitic DQ^{2+} was replaced by methylviologen (MV^{2+}). As the reduction potential of MV^{2+} ($E^0 = -0.44 \text{ V}$) is comparable to that of PVS ($E^0 = -0.41 \text{ V}$), the driving force for interfacial electron exchange is absent. Figure 2a shows that with the $\text{Ru}(\text{bpy})_3^{2+}$ - MV^{2+} -Y, the rate of PVS radical formation is similar to the $\text{Ru}(\text{bpy})_3^{2+}$ -Na-Y, confirming that the directional interfacial electron transfer between DQ^{2+} and PVS is necessary for higher yields of photochemical charge separation. A control experiment using DQ^{2+} -zeolite-Y and PVS without any sensitizer in the zeolite resulted in no viologen radical formation.

Intrazeolitic oxidative electron transfer between $\text{Ru}(\text{bpy})_3^{2+}$ and MV^{2+} occurs readily^{19,21}. It is expected that a similar reaction occurs with DQ^{2+} , resulting in the formation of $\text{Ru}(\text{bpy})_3^{3+}$.

The above experiments were done with a pellet of zeolite sample, and therefore, only the surface particles participated in the photochemical reaction. To estimate the photochemical efficiency of the process, we needed to ensure that all the zeolite particles had access to the radiation. This was done by photolysing a stirred, powdered suspension of $\text{Ru}(\text{bpy})_3^{2+}$ - DQ^{2+} -Y, and estimating the PVS $^{\cdot-}$ in the solution after centrifugation. The growth of the viologen radical as a function of time is plotted in Fig. 3. The rate of formation corresponds to 0.12 mol viologen radical hr^{-1} per mole of $\text{Ru}(\text{bpy})_3^{2+}$ with illumination of 200 mW (420–680 nm). Thus, in the optimum arrangement,

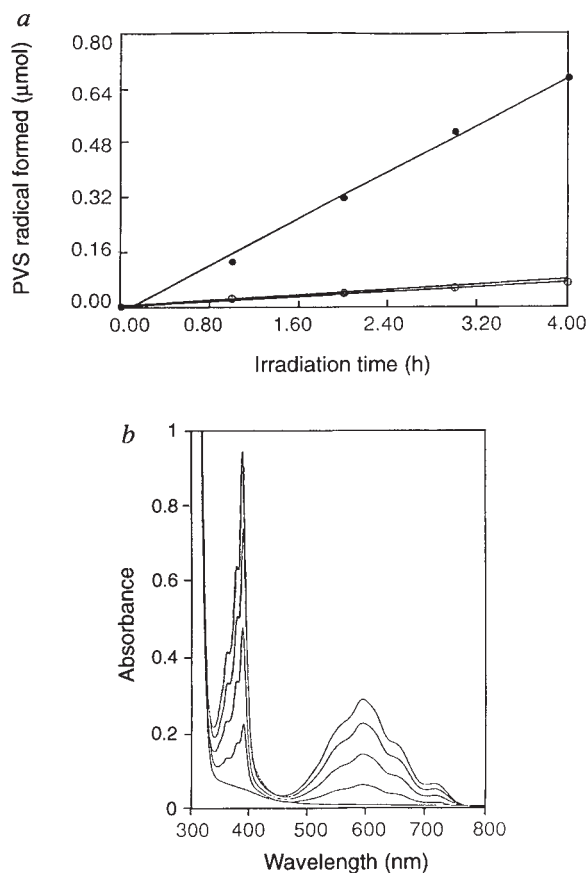


FIG. 2 a, Growth of the viologen radical as a function of photolysis time. 200 mW of 420–680 nm radiation is incident on a 20-mg pellet of $\text{Ru}(\text{bpy})_3^{2+}$ -Y suspended in 3 ml of 0.01 M PVS solution in a 1-cm anaerobic cuvette. (●) zeolite with DQ^{2+} ; (+) zeolite with MV^{2+} ; (○) zeolite with Na^+ (solid lines merely connect the points). b, Observed electronic spectra as a function of photolysis time (every 60 min) for the $\text{Ru}(\text{bpy})_3^{2+}$ - DQ^{2+} -Y with 0.01 M PVS, with conditions same as a.

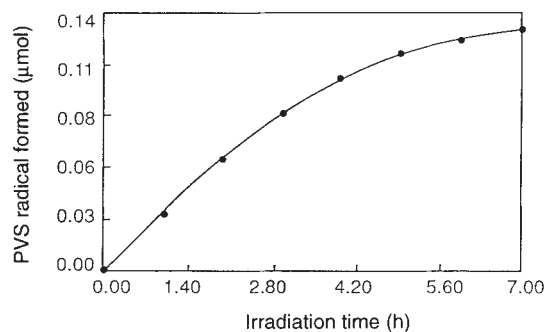


FIG. 3 Growth of the viologen radical during photolysis of a stirred suspension of 5 mg of $\text{Ru}(\text{bpy})_3^{2+}$ - DQ^{2+} -Y in 1 ml of 0.01 M PVS in a sealed tube. (Conditions same as in Fig. 2; solid line merely connects the points).

the rate of growth of the viologen per hour corresponds to ~12% of the sensitizer present. The system approaches a steady state as the radical yield reaches ~60% of the total sensitizer concentration. As the concentration of the viologen radical in the solution increases, absorption of photons by the sensitizer in the sample decreases because of an inner filter effect and is the likely cause of the gradual decrease in the rate of radical formation.

A more quantitative measure of the efficiency of this process is via the quantum yield. This is difficult to determine because the scattering from the zeolite particles interferes with measurement of the light absorbed by the $\text{Ru}(\text{bpy})_3^{2+}$ in the zeolite. Addition of refractive-index-matching solutes such as sucrose to alleviate this problem can interfere with the photoredox process^{22,23}. Therefore, to calculate the light absorbed by the $\text{Ru}(\text{bpy})_3^{2+}$ in zeolite Y, we compared the light transmitted by it to a sample of Na-Y and used the difference to estimate the number of photons absorbed. The quantum yield using laser excitation at 457.9 nm over a period of 30 min was estimated to be 5×10^{-4} . This should be considered an approximate measure. A major factor in this low quantum yield could be the zeolite particle size. The Ru centre is oxidatively quenched by the neighbouring viologens fairly efficiently²¹. In that case, the competition to charge separation arises from the geminate recombination between $\text{Ru}(\text{bpy})_3^{3+}$ and DQ^{+} . Providing an alternate pathway for the electron to transfer from DQ^{+} to PVS at the zeolite-solution interface, as shown here, increases the charge separation efficiency. However, for DQ^{+} molecules that are generated in the internal cages of the zeolite, the charge must propagate to the surface of the particle (by means of electron hopping on intrazeolitic viologen molecules²¹), in direct competition with the back electron transfer reaction, and can be the bottleneck to efficient charge separation. To enhance the quantum yield, the distance from the Ru centre in the zeolite to the PVS in solution will need to be minimized.

Despite the low efficiency, comparison to previous studies⁶⁻¹² indicates several advantages of the present system, including the simplicity of its design. In addition, the electron transfer is mediated by an electron relay that completely surrounds the sensitizer and results in efficient quenching. The ultimate electron acceptor is free in solution and separated physically from the donor, providing ready access to the viologen redox products. Current work is focusing on other intrazeolitic and external viologens, the role of zeolite particle size and strategies to gain access to the photoredox ruthenium species in the zeolite. □

²³¹Pa/²³⁰Th ratios in sediments as a proxy for past changes in Southern Ocean productivity

N. Kumar*, R. Gwiazda*, R. F. Anderson & P. N. Froelich*

Lamont-Doherty Earth Observatory of Columbia University, Palisades, New York 10964, USA

THE biological productivity of the oceans is sensitive to changes in climate, which can affect essential factors such as nutrient and light availability. In turn, ocean productivity may influence climate by regulating the partitioning of carbon dioxide, a greenhouse gas, between the ocean and the atmosphere. Investigators have attempted to link variations in atmospheric CO_2 content, recorded in ice cores^{1,2}, to the productivity of the Southern Ocean³⁻⁶, but an unambiguous means of assessing past changes in ocean productivity has been lacking. Here we exploit established relationships between ²³¹Pa/²³⁰Th ratios and particle flux⁷⁻¹² to infer, from the analysis of dated sediment cores, variability through time of fluxes of particulate biogenic material exported from surface waters. Records from two cores in the Atlantic sector of the Southern Ocean indicate that ocean productivity during glacial periods was lower than at present south of the Antarctic polar front, and support earlier conclusions¹³⁻¹⁶ that the zone of maximum productivity migrated northwards during glacial conditions. Although further work at other sites is needed for an assessment of changes in total Antarctic productivity, our technique has the potential to provide this information while avoiding some of the limitations of other productivity proxies.

Changes in ocean productivity can be inferred from known relationships between the isotope (for example $\delta^{13}\text{C}$) or trace element (for example Cd/Ca) content of planktonic foraminifera and the nutrient content of the waters in which they grew, assuming that an increase in productivity will be accompanied by a decrease in nutrient concentration if all other factors remain unchanged. Attempts to reconstruct nutrient contents of glacial Southern Ocean surface waters have produced ambiguous results, however, in that foraminifera $\delta^{13}\text{C}$ is lower during glacial times (implying higher nutrient concentrations)^{17,18}, whereas Cd/Ca ratios suggest little systematic change in surface Cd concentration, much less than expected from polar CO_2 models^{19,20}. Ge/Si ratios of diatoms cannot be used as a proxy for surface water nutrient concentrations, as previously proposed^{13,21}, because seawater Ge/Si is not fractionated during diatom shell formation²².

Past changes in productivity may also be assessed, in principle, from the record of biogenic detritus buried in the sea bed. Export of organic carbon and nutrients to the deep sea is driven largely by diatoms in the Southern Ocean, so opal accumulation rates are expected to reflect the productivity of overlying surface waters^{13,14}. But interpretation is hampered by sediment focusing and by our limited ability to quantify opal dissolution²³. Berger speculated²⁴, furthermore, that dissolved silica may have become a limiting nutrient in glacial polar surface waters, decoupling the C and Si fluxes, and permitting a high flux of particulate organic matter to have been generated by nonsiliceous phytoplankton.

The mass flux of particles through the water column varies with primary productivity²⁵, so we can avoid these complications by using tracer that responds to changing particle flux. Radionuclides from the uranium decay series provide such a proxy. ²³¹Pa ($t_{1/2} = 32,500$ yr) and ²³⁰Th ($t_{1/2} = 75,200$ yr) are produced in sea water by decay of dissolved ²³⁵U and ²³⁴U, respectively. The

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* Also at Department of Geological Sciences, Columbia University.

salinity-normalized concentration and the isotopic composition of U in seawater are spatially uniform^{26,27}, reflecting the long residence time of U in the oceans²⁸, and provide a uniformly distributed source of ^{231}Pa and ^{230}Th with a constant initial Pa/Th activity ratio of 0.093. Pa and Th are among the most particle-reactive elements in the ocean; their mean oceanic residence times, with respect to scavenging to the sea floor, are ~ 10 – 100 yr (ref. 29). Because their residence times in the ocean are much less than their radioactive half-lives, virtually all of the ^{231}Pa and ^{230}Th produced in sea water is removed to sediments.

Investigators have previously used the well-defined source of ^{230}Th to evaluate time-varying fluxes of particulate materials^{30,31} on the principle that the flux of ^{230}Th from the water column is equal to its rate of production by U decay, which is a simple function of water depth (see Fig. 2 legend). Although this approach permits the evaluation of fluxes in regions where sediment focusing leads to inhomogeneous patterns of sediment accumulation, it is limited by the fact that dissolution of labile biogenic phases also influences the ratios (C_s/A_{Th} ; see Fig. 2) preserved in the sediments³². The calculated fluxes are referred to here as 'preserved fluxes'.

$^{231}\text{Pa}/^{230}\text{Th}$ ratios provide an alternative record of changes in particle flux that is insensitive both to dissolution of biogenic phases at the sea floor and to the effects of sediment focusing. Although both ^{231}Pa and ^{230}Th are quantitatively scavenged to sediments, Th is more particle-reactive than Pa (refs 7, 8). The residence time of Th is short compared with the timescales of lateral mixing in the deep sea, so the flux of scavenged particulate

^{230}Th from the water column roughly balances its local rate of production by U decay^{9,31,33}. ^{231}Pa remains in the water column long enough to be transported over basin-scale distances between its production in the deep sea and its removal to sediments. Consequently, fluxes of particulate ^{231}Pa scavenged from the water column vary as a function of particle flux (scavenging intensity)^{9–11}, so particulate $^{231}\text{Pa}/^{230}\text{Th}$ ratio increase with increasing particle flux. The ratios may exceed the production ratio of 0.093 in regions of high particle flux by a factor of three, whereas sediments in oligotrophic central ocean gyres typically have $^{231}\text{Pa}/^{230}\text{Th}$ ratios less than half the production ratio^{7,9,10,12}. Although the composition of particulate matter influences the partitioning of Pa between solution and particulate phase⁸, the mass flux of particles settling through the water column seems to be the principal factor determining particulate $^{231}\text{Pa}/^{230}\text{Th}$ ratios^{10,11}. Extending the results for the modern ocean to the time domain provides the basis for a new proxy of past changes in ocean productivity.

We chose cores RC13-259 ($53^\circ 53' \text{S}$, $4^\circ 56' \text{W}$, 2,677 m water depth) and RC13-254 ($48^\circ 34' \text{S}$, $5^\circ 07' \text{E}$, 3,636 m water depth) for this study because their chronostratigraphy and opal accumulation records are well characterized (refs 13, 14; Fig. 1). Patterns of sediment accumulation recorded in RC13-259 are representative of the zone just south of the present position of the Antarctic polar front (APF)^{13,14}. Opal burial rates at this

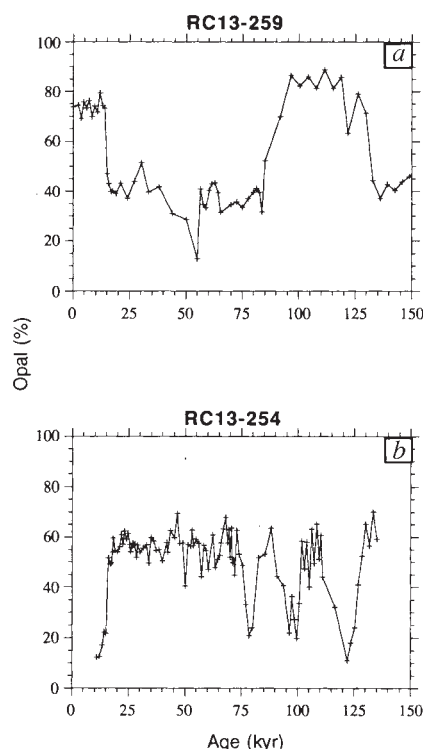


FIG. 1 Opal records¹⁴ show that sediments deposited south of the present position of the Antarctic polar front (a) exhibit patterns of low-glacial/high-interglacial opal contents, whereas the opposite pattern is observed in the region $\sim 5^\circ$ further north (b). To help illustrate temporal relationships between changes observed at each site, and with the record of global climate change, results have been plotted against age using the core chronologies of Charles *et al.*¹⁴ Chronologies of ^{18}O stages 2–4 are not well constrained in either core, but the age uncertainty does not obscure the main changes in sediment accumulation that occurred during transitions between glacial and interglacial conditions.

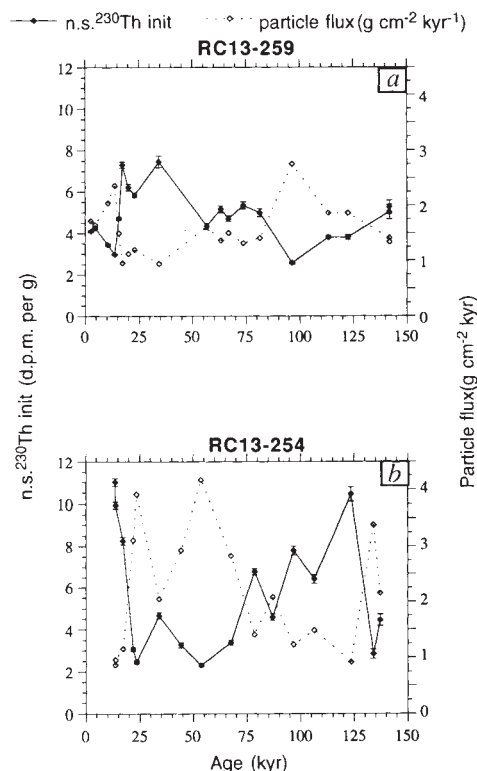


FIG. 2 Initial unsupported ^{230}Th activities in sediments of RC13-259 (a), located south of the present position of the APF, and RC13-254 (b), located north of the APF, are plotted against age, along with the preserved flux of particles computed by the ^{230}Th -profiling method. The flux of ^{230}Th from the water column (F_{Th}) is equal to its rate of production by U decay (P_{Th}), which is related to water depth Z (in metres) by P_{Th} (d.p.m. $\text{cm}^{-2} \text{kyr}^{-1}$) = $2.6 \times 10^{-3} Z$. Fluxes of other particulate substances (F_s) can then be estimated as $F_s = C_s/A_{\text{Th}} \times P_{\text{Th}}$, where C_s and A_{Th} are the concentration of the substance or phase of interest and the initial (decay-corrected) unsupported ^{230}Th activity of the sediments, respectively. ('unsupported' here means produced by U decay in the water column). Unsupported ^{230}Th activities are decay-corrected to the time of deposition using core chronologies described in Fig. 1.

site are high during interglacial periods ($\sim 2.4 \text{ g cm}^{-2} \text{ kyr}^{-1}$ during the Holocene), but low during glacial ($\sim 0.5 \text{ g cm}^{-2} \text{ kyr}^{-1}$ during the Last Glacial Maximum, LGM). The more northerly location of RC13-254 shows the reverse pattern, with minimum opal accumulation ($< 0.5 \text{ g cm}^{-2} \text{ kyr}^{-1}$) during peak interglacials and maximum accumulation during glacials (average $> 2 \text{ g cm}^{-2} \text{ kyr}^{-1}$ during ^{18}O stages 2–4; refs 13, 14). These features are interpreted as reflecting the increase of Southern Ocean sea-ice cover during glacial periods which, in turn, drives northward the zone of high siliceous productivity^{15,16}.

We re-examined sediment accumulation rates using the ^{230}Th -profiling technique to remove effects of sediment focusing. Measured ^{230}Th activities were corrected for detrital and authigenic U-supported components (N.K., manuscript in preparation), and then decay-corrected to time of deposition using published core chronologies¹⁴. Initial unsupported (n.s.) ^{230}Th activities at the southerly location of RC13-259 (Fig. 2a) show their greatest change, a decrease by more than a factor of two, from the LGM into the early Holocene, implying that the preserved flux of opal-rich sediments increased here by more than a factor of two at this time. Deeper in the core, initial n.s. ^{230}Th varies less than at the LGM–Holocene boundary, but the patterns are consistent with generally higher preserved fluxes during interglacials. Larger changes are recorded at the northerly location of RC13-254 (Fig. 2b), where initial n.s. ^{230}Th activities indicate a fourfold decrease in preserved flux across the transition from LGM to early Holocene. A similar change is evident at termination II ($\sim 130 \text{ kyr}$ before present, BP).

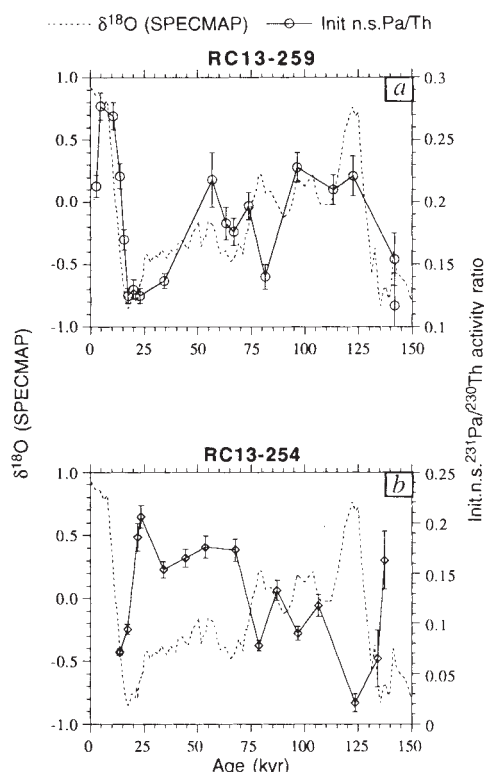


FIG. 3 Initial unsupported $^{231}\text{Pa}/^{230}\text{Th}$ activity ratios in sediments of RC13-259 (a), located south of the present position of the APF, and RC13-254 (b), located north of the APF, are plotted against age along with the SPECMAP $\delta^{18}\text{O}$ record³⁷ which is shown to illustrate glacial–interglacial cycles over this time interval. Throughout the ocean, $^{231}\text{Pa}/^{230}\text{Th}$ ratios tend to increase with increasing particle flux. Extending this relationship to the time domain, we interpret the minimum (maximum) values in these records to reflect times of lower (greater) particle flux and, therefore, lower (greater) ocean productivity. Unsupported ^{231}Pa and ^{230}Th activities are decay corrected to the time of deposition using core chronologies described in Fig. 1.

We subsequently made an independent assessment of past changes in patterns of particle flux using the $^{231}\text{Pa}/^{230}\text{Th}$ -ratio proxy. Initial n.s. ^{231}Pa activities were computed as described above for ^{230}Th . South of the APF (RC13-259), initial n.s. $^{231}\text{Pa}/^{230}\text{Th}$ ratios vary by more than a factor of two (Fig. 3a); minimum values, implying intervals of diminished particle flux (lower productivity), coincide with glacial maxima (stages 2 at $\sim 20 \text{ kyr}$ and 6 at $\sim 140 \text{ kyr}$). North of the APF (RC13-254), initial n.s. $^{231}\text{Pa}/^{230}\text{Th}$ ratios vary by more than a factor of three (Fig. 3b), but the pattern of variability is opposite to that observed south of the APF. During peak interglacials, initial n.s. $^{231}\text{Pa}/^{230}\text{Th}$ ratios are well below the seawater production ratio of 0.093, implying a low particle flux typical of oligotrophic central ocean gyres^{7,10,12}. Much greater glacial particle fluxes in this region, implied by the ^{230}Th -profiling results (Fig. 2b), are confirmed by the $^{231}\text{Pa}/^{230}\text{Th}$ ratios which exceed the production ratio by about a factor of two during peak glacial (Fig. 3b).

These results allow us to make an interpretation of past changes in Southern Ocean productivity. The ^{230}Th -profiling method and the use of $^{231}\text{Pa}/^{230}\text{Th}$ ratios as a proxy of palaeo-particle flux provide records that are not only internally consistent but are also consistent with interpretations based on mass accumulation rates of biogenic silica^{13,14}. South of the present APF (RC13-259), productivity (as reflected by opal accumulation and by particle flux) was lower during glacial than during interglacials, regardless of whether the sinking flux of particles was created by diatoms or by other organisms²⁴. North of the APF (RC13-254), on the other hand, productivity peaked during glacial maxima and subsided to low levels typical of oligotrophic gyres during interglacials. Our results support earlier conclusions^{13–16}, that the zone of maximum productivity migrated north of its present position during glacial times, although results from just two sites are insufficient, in themselves, to estimate net glacial-to-interglacial changes in the productivity of the entire Southern Ocean.

The use of $^{231}\text{Pa}/^{230}\text{Th}$ ratios to monitor past changes in ocean productivity is not restricted to the Southern Ocean. Several studies^{34–36} have concluded that productivity was higher during glacial times in regions of wind-driven coastal upwelling and in the equatorial Atlantic and Pacific Oceans. Each study has recognized, however, that variable preservation of biogenic materials limits the ability to infer past changes in ocean productivity. Regional mapping of $^{231}\text{Pa}/^{230}\text{Th}$ ratios in glacial-age sediments could provide an independent test of hypotheses about climate-driven changes in patterns of ocean productivity. □

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Accelerated soil erosion around a Mexican highland lake caused by prehispanic agriculture

Sarah L. O'Hara*, F. Alayne Street-Perrott†‡
& Timothy P. Burt‡

* Department of Geography, University of Sheffield, Western Bank, Sheffield S10 2TN, UK

† Environmental Change Unit, 1a Mansfield Road, Oxford OX1 3TB, UK

‡ School of Geography, Mansfield Road, Oxford OX1 3TB, UK

THE severely degraded landscape of the volcanic highlands of central Mexico has been the focus of considerable debate^{1–5}. Although it is widely believed that the Spanish encountered an almost pristine landscape in AD 1521 (refs 1–3), some archival and palaeolimnological studies have suggested that extensive land clearance began before European contact, during the Preclassic to Postclassic periods (~3,500–350 ¹⁴C yr before present, BP)^{5–11}. Here we analyse sediment cores from Lake Pátzcuaro, Michoacán (Fig. 1), to derive a quantitative estimate of variations in soil erosion in central Mexico since 4,000 yr BP. We identify three periods of accelerated erosion and conclude that erosion rates during both the late Preclassic/early Classic periods (2,500–1,200 yr BP) and the later Postclassic period (850–350 yr BP) were at least as high as those after the Spanish conquest. One implication of these results is that soil erosion caused by the Spanish introduction of plough agriculture was apparently no more severe than that associated with traditional agricultural methods; it is therefore questionable whether a return to traditional methods would have significant environmental benefits.

Lake Pátzcuaro lies in a small (927 km²), closed intermontane basin in the volcanic highlands of Michoacán (Fig. 1). The lake (126 km²; 2,036 m above sea level (a.s.l.); ~1,000 mm annual precipitation) is eutrophic to hypereutrophic and can be divided into south, central and north basins, with maximum water depths increasing northwards from 1–2 m to 12 m; the average depth is 4.6 m. Suspended sediment concentrations also increase northwards, probably because of current drift¹². Bordering the lake there are thick deposits of reddish, charcoal-rich colluvium¹¹. The surrounding volcanic cones are gently sloping with few gullies to the south, but increasingly steep and heavily gullied towards the north. Thorn scrub is widespread on the lower slopes, although the upper slopes still support secondary pine-oak woodlands. Palaeoclimatic reconstructions for Pátzcuaro and adjacent areas are given in refs 6–8, 13–16.

The archaeology of this region is poorly documented. Immediately before the Spanish Conquest in AD 1521, the Pátzcuaro basin was the focal point of the Postclassic Purépecha (Tarascan) Empire, with an estimated population of 60,000–105,000 (ref. 17), of whom ~35,000 lived in Tzintzuntzan on the northeast shore¹⁸ (Fig. 1). Judging from the *Relación de Michoacán*¹⁹, the Purépecha were obsessed by the need to collect wood, primarily

for ceremonial bonfires, but also for the production of beams, boards and other lumber¹⁹. Evidence of sedentary agriculture in the basin before the Purépecha arrived ~1,000 yr BP (ref. 18) is derived largely from the presence of maize (*Zea mays*) and weedy species in the pollen record from the early Preclassic (3,500 yr BP) onwards⁶. During the late Preclassic/early Classic period the region may have come under the influence of the Chupícuaro, who occupied agricultural and craft centres with small pyramids²⁰.

Data on accelerated soil erosion in the tropics is generally derived from small experimental plots, with only a few years of data. Such studies may yield exaggerated estimates of total soil loss³⁰. Hence it is difficult to derive accurate and spatially-representative predictions of the impact of future land-use change or to examine the long-term stability of lake ecosystems in the face of repeated episodes of accelerated nutrient input. The analysis of an array of radiocarbon dated lake-sediment cores provides an alternative means of assessing the sustained impact of deforestation on a large catchment scale.

To evaluate long-term variations in soil erosion in the Pátzcuaro basin we collected 20 sediment cores (1.24–2.85 m in length) from the lake in 1988 using a simple gravity-percussion corer¹⁵ (Fig. 1). Sixty-four profiles of colluvium were logged and sampled. In addition, we took samples from the upper 3.36 m of a 14.2-m core (the Mastercore) previously analysed for pollen⁶ and diatoms (J. P. Bradbury, unpublished data). The lake sediments consist of calcareous organic gyttja, ostracode-rich silts and clays. On the basis of stratigraphy, bulk density, magnetic susceptibility (χ), loss-on-ignition, carbonate content and sediment chemistry, six main lithological units can be identified (Fig. 2)¹⁵.

The organic gyttja (unit I) and ostracode-rich layers (units III and V) are characterized by high organic and carbonate concentrations but low χ values, suggesting relative stability within the catchment. In marked contrast, the clay units (II, IV and VI) contain little organic matter or carbonate and exhibit large peaks in χ , inorganic Si and cations associated with volcanic soils (Fe, Al, Ti and so on)¹⁵. We infer that they accumulated during periods of accelerated soil erosion. Clayey deposits are not found below unit I (refs 6, 15) implying that climatic change alone cannot account for these sediments.

The first, short-lived erosional event (unit II) is represented by a small peak in χ in the south basin, where the accumulation rate of sediment, averaged over the relevant Thiessen polygons, was $29.2 \pm 13.6 \text{ g m}^{-2} \text{ yr}^{-1}$ (Table 1a). It is bracketed by dates

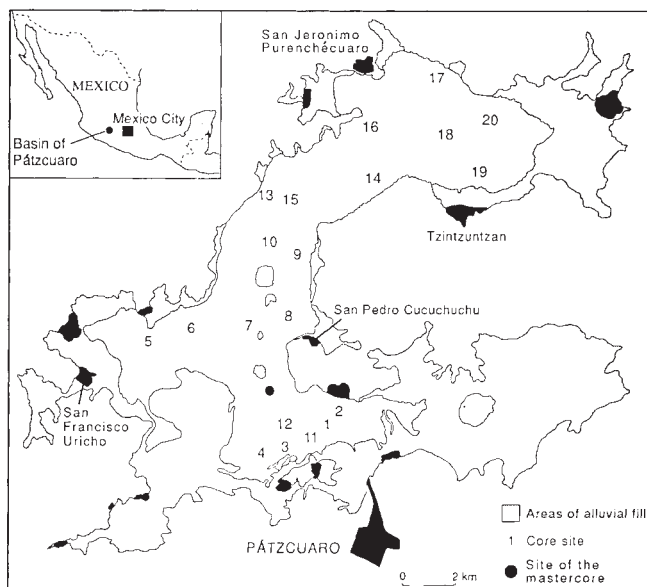


FIG. 1 Location of Lake Pátzcuaro and lake sediment cores.

TABLE 1 Sediment influx, phosphorus influx and dating control for selected sediment cores from Lake Pátzcuaro

a, Influx and yield of phosphorus and inorganic sediment													
SOUTH BASIN				SOUTH BASIN		CENTRAL BASIN		NORTH BASIN		Estimated input		Estimated yield	
LP11				Mastercore		LP10		LP19		to lake		from catchment to lake	
influx				influx		influx		influx					
		Sediment	P	Sediment	P	Sediment	P	Sediment	P	Sediment	P	Sediment	P
Unit	Time span	(g m ⁻²	(g m ⁻²	(g m ⁻²	(g m ⁻²	(g m ⁻²	(g m ⁻²	(g m ⁻²	(g m ⁻²	(10 ⁶ g	(10 ⁶ g	(10 ³ g	(10 ³ g
	(yr)	yr ⁻¹)	yr ⁻¹)	yr ⁻¹)	yr ⁻¹)	yr ⁻¹)	yr ⁻¹)	yr ⁻¹)	yr ⁻¹)	yr ⁻¹)	yr ⁻¹)	ha ⁻¹ yr ⁻¹)	ha ⁻¹ yr ⁻¹)
VI	850	118	0.11	153	0.79	130	0.15	553	0.30	29,000	32.4	362	0.41
V	350	40	0.07	37	0.48	77	0.16	66	0.25	4,400	26.4	55	0.33
IV	1,300	49	0.7	115	0.28	162	0.31	131	0.10	10,300	18.3	129	0.23
III	550	41	0.13	47	0.10	NA	NA	NA	NA	(3,700)	(20.0)	(46)	(0.23)
II	550	46	0.07	76	0.15	NA	NA	NA	NA	(5,100)	(20.4)	(64)	(0.25)
I	450	51	0.12	47	0.23	NA	NA	NA	NA	(4,100)	(19.3)	(51)	(0.26)

b, Radiocarbon chronology					
Unit	Site	¹⁴ C age (yr BP)	Lab. No.	Material dated*	Refs
VI	A	Modern	SRR-1860	Charcoal ^a	11
VI	A	Modern	SSR-1861	Charcoal ^a	11
VI	B	90 ± 60	OxA-2852	Charcoal ^a	
VI	B	180 ± 60	OxA-2853	Charcoal ^a	
VI	A	270 ± 90	SRR-1859	Charcoal ^a	11
VI	B	270 ± 60	OxA-2851	Charcoal ^a	
VI	LP12	480 ± 60	OxA-2822	Mixed shell ^b	
VI	C	740 ± 100	OxA-2825	Mixed shell ^b	
V	LP19	880 ± 60	OxA-2823	Ostracodes ^b	
V	LP11	1,190 ± 70	OxA-2817	Ostracodes ^b	
IV	LP5	1,580 ± 60	OxA-2820	Ostracodes ^b	
IV	A	2,300 ± 60	SRR-1862	Bulk organic ^c	6
III	LP19	2,530 ± 60	OxA-2825	Ostracodes ^b	
III	LP11	2,650 ± 60	OxA-2818	Ostracodes ^b	
III	Mastercore	2,890 ± 80	QL-1341	Bulk organic ^b	6
III	LP5	2,920 ± 80	OxA-2821	Ostracodes ^b	
I	Mastercore	3,640 ± 80	QL-1342	Bulk organic ^b	6
I	LP11	3,640 ± 70	OxA-2819	Ostracodes ^b	

a, Variations in the influx of inorganic sediment and phosphorus to Lake Pátzcuaro and estimated outputs from the catchment, 4,000–0 yr BP. Sediment influx was assessed on an organic-, carbonate- and biogenic opal-free, dry-weight basis. Results from the array of 21 sediment cores (in $\text{g m}^{-2} \text{ yr}^{-1}$) were scaled up to the entire lake using Thiessen polygons²⁸. Total sediment influxes for units I, II and III (in parentheses) are estimated on the basis of cores from the south basin only. Outputs from the catchment only cover sediment accumulation in the lake. A first-order estimate suggests that ~40% of sediment eroded from the slopes reached the lake; the remaining 60% was stored as colluvium in marginal embayments (Fig. 1). Phosphorus concentrations were measured only at the four sites listed. Total P influxes for units I, II and III (in parentheses) were estimated from the two south basin cores. b, Radiocarbon chronology of erosional episodes in the Basin of Pátzcuaro ~4,000–0 yr BP. All ^{14}C dates are uncalibrated. OxA dates were measured by accelerator mass spectrometry, other dates are conventional ages. There are no limestones or volcanic springs in the catchment.

Areas (see Fig. 1). A, San Jerónimo Purenchécuaro; B, San Francisco Uricho; C, San Pedro Cucuchuchu.

* Facies: a, Colluvium; b, Lacustrine; c, Lacustrine/marsh.

of $3,640 \pm 80$ and $2,890 \pm 80$ yr BP in the Mastercore (Table 1b). Its onset coincides with the first appearance of *Zea* pollen^{6,15}. The cores from the central and north basins are too short to record this event. A similar erosional episode at La Piscina de Yuriria (50 km northeast of Pátzcuaro) is also associated with the first appearance of maize ~3,200 yr BP (ref. 16).

A second, more intense period of erosion (unit IV) is bracketed by dates of $2,530 \pm 60$ and $1,190 \pm 70$ yr BP (Table 1b). Based on the full set of 21 cores, an estimated 13.4 Mt of sediment were deposited in the lake at an average rate of $10,300 \text{ t yr}^{-1}$ between 2,500 and 1,200 yr BP (Table 1a). The average sediment accumulation increased from $33.8 \pm 37.5 \text{ g m}^{-2} \text{ yr}^{-1}$ in the south basin to 120.4 ± 64.3 and $154.7 \pm 84.2 \text{ g m}^{-2} \text{ yr}^{-1}$ in the central and north basins, respectively. This suggests that the main late Pre-classic/early Classic occupation sites were in the northern part of the basin and that the thick deposits of colluvium that border this part of the lake have buried their remains. Early agriculturalists may have preferred to farm the steeper slopes for two reasons: the drainage channels could be dammed for irrigation^{21,22}, and the risk of frost damage to crops would be minimized²³. The sediment geochemistry¹⁵, which will be discussed in full elsewhere, implies that erosion of subsoil (fraction c in Fig. 2), possibly by gully, was more important than the removal of fine weathered topsoil (fraction a).

A period of catchment stability occurred in the late

Classic/early Postclassic between ~1,200 and 850 yr BP, during which the sediment influx to the lake dropped sharply to less than $4,500 \text{ t yr}^{-1}$ (Table 1a). Sediment accumulation rates decreased in the north ($58.0 \pm 24.5 \text{ g m}^{-2} \text{ yr}^{-1}$) and central basins ($44.4 \pm 16.6 \text{ g m}^{-2} \text{ yr}^{-1}$), but changed little in the south basin ($41.7 \pm 15.1 \text{ g m}^{-2} \text{ yr}^{-1}$). The region suffered a prolonged drought during this interval^{8,14–16} when the level of Lake Pátzcuaro fell by ~4–5 m (ref. 15) and the basin may have been abandoned.

Renewed erosion began in the Postclassic about 850 yr BP (unit VI) shortly after the arrival of the Purépecha. An estimated 24.5 Mt of sediment have been deposited in the lake since this date at an average rate of $29,000 \text{ t yr}^{-1}$ (Table 1a). Accelerated erosion was more widespread than in the second episode, reflecting extensive forest clearance by the Purépecha. However, average sediment accumulation rates were again higher in the north ($329.4 \pm 164.3 \text{ g m}^{-2} \text{ yr}^{-1}$) than the central ($160.0 \pm 61.3 \text{ g m}^{-2} \text{ yr}^{-1}$) and south basins ($146.0 \pm 22.4 \text{ g m}^{-2} \text{ yr}^{-1}$), in part because of the concentration of settlement around Tzintzuntzan (Fig. 1) during the late Postclassic.

It is hard to distinguish any specific impact of the introduction of plough agriculture and draught animals by the Spanish after AD 1521. Comparison of the sediment influx above and below a date of 480 ± 60 yr BP (AD 1405–1450; ref. 24) at 30 cm in core 12 (Fig. 3) indicates that if anything there was a decrease in the erosion rate after the Conquest. A sharp drop in population¹⁷

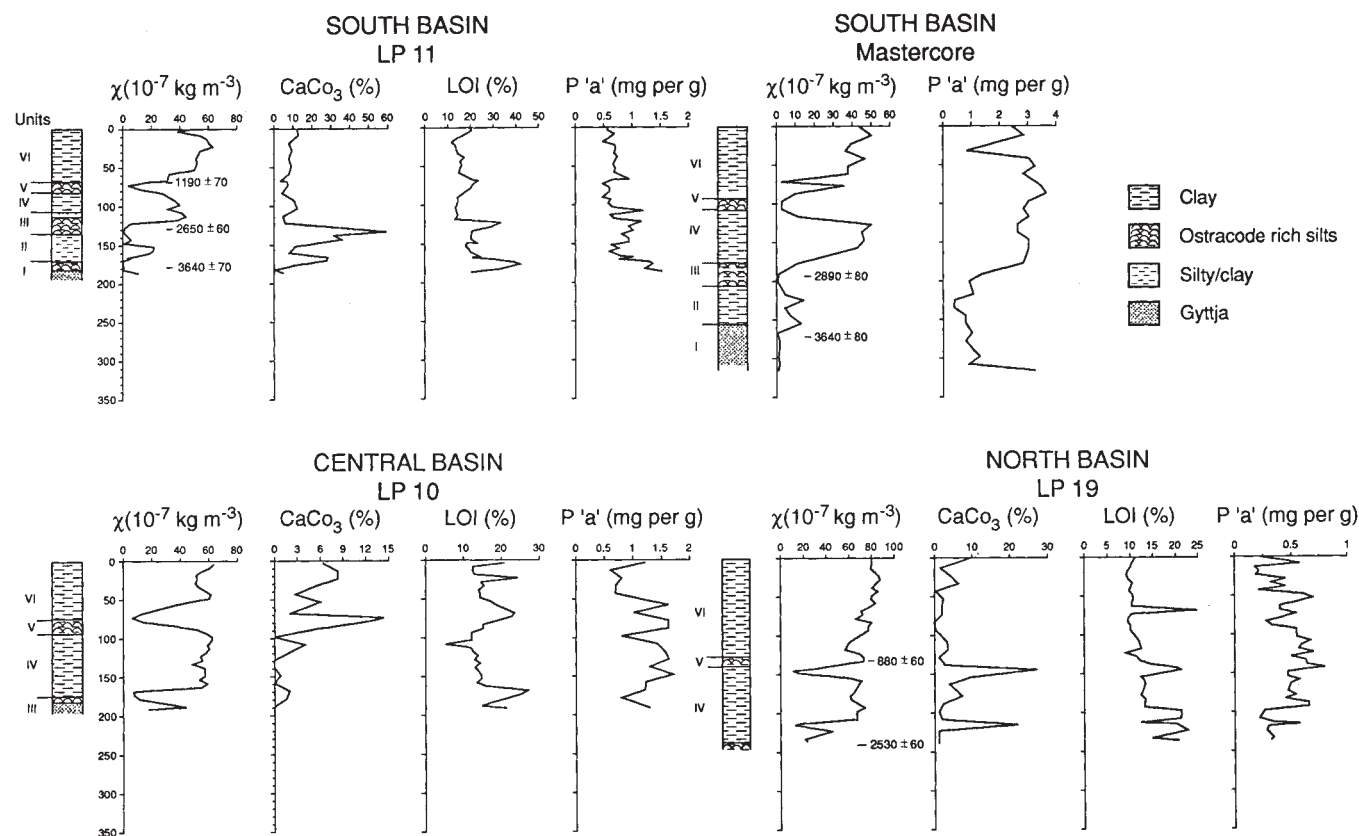


FIG. 2 Magnetic susceptibility (χ), carbonate content, loss-on-ignition (LOI) and phosphorus ('a' fraction) for selected cores from Lake Pátzcuaro. Analyses were made at 5–10 cm intervals. χ was measured using a Bartington MS1 susceptibility meter. LOI was determined by ignition at 550 °C for 1 h. Carbonate content was measured manometrically by reaction with dilute HCl. Samples for sediment chemistry were prepared using a wet-chemical

extraction technique²⁹. This separates the sediment into an H_2O_2 -HCl-soluble 'a' fraction, a NaOH-soluble biogenic opal 'b' fraction and a residual 'c' fraction. The 'a' fraction includes organic matter, carbonates, and labile oxides and oxyhydroxides derived from topsoil. The 'c' fraction consists largely of unweathered detrital minerals. Major cations were determined by flame atomic absorption, and P and Si by automated colorimetry.

coupled with forest regeneration¹⁵ in the Basin of Pátzcuaro may have significantly reduced erosion. However, a shift in the type of sediment from weathered to unweathered material is noted at this level. This may have resulted from a number of factors: progressive stripping of the upper soil horizons, gullyng or the introduction of plough agriculture.

Spatial and temporal variations in phosphorus accumulation rates were more complex than those of mineral soil, reflecting changes in erosional processes, recycling within the lake and the input of human effluent from specific point sources. Phosphorus accumulation reached a maximum during the deposition of unit VI (Table 1a) when the *per capita* output of 0.31–0.54 kg yr⁻¹ was similar to that reported for the Classic Maya²⁵. The prevalence of *Stephanodiscus* sp. in the diatom record is further evidence for cultural eutrophication since ~900 yr BP (J. P. Bradbury, unpublished data).

This investigation indicates the long-term impact of indigenous farming systems on a tropical lake. The environmental impact of precolonial agriculture in the subhumid highlands of central México equalled that of the Classic Maya cities in the lowland rainforests of Guatemala^{25,26}, and was even more severe than that of the indigenous cultivators in New Guinea²⁷. There is a move by many environmental agencies both in Mexico and elsewhere for a return to traditional forms of agriculture, as they are considered to be better for the environment. As our findings indicate that traditional farming techniques cause significant erosion, it is unlikely that a return to prehispanic farming methods would solve the problem of environmental degradation.

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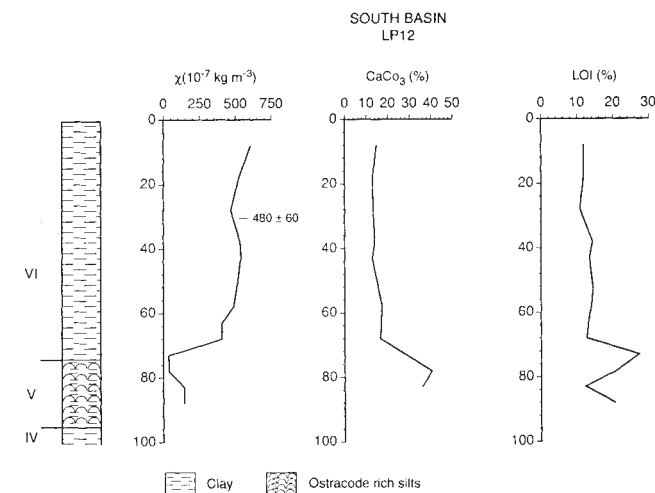


FIG. 3 Magnetic susceptibility (χ), carbonate content and loss-on-ignition (LOI) for core LP12, Lake Pátzcuaro.

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Longitudinally confined geomagnetic reversal paths from non-dipolar transition fields

David Gubbins* & Robert S. Coe†

* Department of Earth Sciences, Leeds University, Leeds LS2 9JT, UK

† Earth Sciences Board, University of California, Santa Cruz, California 95064, USA

It has long been thought that conditions at the boundary between the core and mantle influence the Earth's magnetic field, but the supporting evidence is rather indirect^{1–3}. Recent palaeomagnetic results, suggesting that there are persistent preferred longitudinal paths for the virtual geomagnetic pole (VGP) during reversals⁴, would provide the first direct evidence of the solid mantle's influence on the core, although their statistical significance has been disputed^{5,6}. The results are potentially exciting because the preferred paths lie close to the Pacific rim, where the present geomagnetic secular variation changes character^{2,7}. Here we present a simple model, based on an extension of a previous theory⁸, that produces reversals with VGP paths confined within relatively narrow longitude bands despite the transition field having a substantially non-dipolar structure. Thus, although longitude bias of the VGP paths is definitive evidence for core–mantle interaction, simple VGP paths are not evidence of near-dipolar transition fields.

Many papers^{5–7,9} discuss the statistical validity of the observational evidence for persistent transition paths. Here we discuss a different aspect of the problem: their possible underlying cause. One of us (D.G.) developed the model described below¹⁰ before it was explained to him by the second author (R.S.C.) that VGPs are plotted in an asymmetrical way that appears natural to the palaeomagnetist but is confusing when considering the theory. We believe that few theoreticians appreciated this point immediately, and therefore discuss it first.

The main use of palaeomagnetic poles is to determine tectonic movement, when it is essential to know whether the rocks have

been stamped by a normal or reversed field. By convention the south magnetic pole, which is now close to the North Geographic Pole, is plotted. However, in the deep Earth, where there is no permanent magnetization, the convention is inappropriate. The dynamo equations, including all the boundary conditions regarded so far as plausible, and the equations governing the magnetic field in that part of the mantle that is above the Curie temperature, are invariant under reversal of sign of the magnetic field. Therefore, if geomagnetic reversal involves the entire field, its sense becomes irrelevant. Consider a transition from normal to reverse ($N \rightarrow R$). We plot the south magnetic pole from its initial position at the North Geographic Pole through, for example, the Americas, to the South Geographic Pole. Now consider the next reversal ($R \rightarrow N$) which takes the south magnetic pole back to the North Geographic Pole. If the sense of the magnetic field is irrelevant, but the same physics applies as for the first reversal, the pole that starts at the North Geographic Pole must once again pass through the Americas. This time, however, the VGP starts from the South Geographic Pole and tracks along the other half of the great circle path, through Asia.

Transition data are therefore more readily interpreted if the magnetic pole lying initially at the North Geographic Pole is plotted regardless of its sign. This has already been pointed out by Hoffman¹¹ but his advice has been ignored. The preponderance of VGP paths lying along the eastern Pacific rim reported by Laj^{6,7} and others is therefore evidence of asymmetry between reversed and normal states in the field. It cannot be explained by conventional theory, including the mechanisms presented here and that of Runcorn¹², unless $N \rightarrow R(R \rightarrow N)$ paths along the E rim are accompanied by $R \rightarrow N(N \rightarrow R)$ paths around the antipodal path. Valet *et al.*⁵ test the hypothesis that VGP paths cluster around preferred longitudes but they also mix $N \rightarrow R$ and $R \rightarrow N$ transitions, thereby partially randomizing their dataset. For $R \rightarrow N$ transitions they should have plotted the north magnetic pole.

There is no theoretical requirement for time symmetry in reversal: it may initiate preferentially in one hemisphere, for example, or may start slowly and finish quickly. Persistent intermediate directions may also be established⁹; averaging effects of the magnetic recording process may then lead to preferred longitudes for VGP paths⁹, but not to N/R asymmetry. We show below that time asymmetry can determine whether the VGP tracks along the eastern or western half of the great circle.

To return to the question of how longitude-confined VGP paths can arise from complex behaviour of the dynamo, we

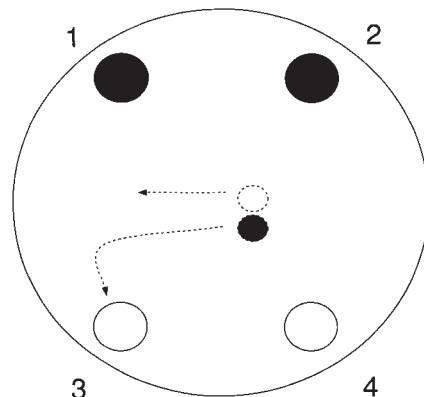


FIG. 1 Flux expulsion creates a pair of flux patches of opposite polarity at the core surface; they are then carried by the core flow. One patch travels southwest to eliminate part of the flux lobe 3 while the other is carried west along the Equator to rest at the Pacific rim, near 90° W. Similar behaviour is observed in the present SV.

propose a simple way to link VGP movements with flux change at the core surface, thereby connecting palaeomagnetic data with intuition gained from secular variation (SV) studies. Core fields show three basic changes: drifting of flux, change in strength of patches of flux, and growth of pairs of positive and negative flux through expulsion of toroidal field through the surface. The latter effect is intimately linked with solar magnetic reversals and is therefore worthy of our attention; it will be detectable palaeomagnetically but lost in the usual low-order spherical harmonic analysis. We simplify field changes at the core surface by shrinking the characteristic flux concentrations to points and writing the vertical field as

$$\sum_i F_i [\delta(\hat{r} - \hat{r}_i^+) - \delta(\hat{r} - \hat{r}_i^-)] \quad (1)$$

where F_i represents the strength of the i th pair of flux patches and $\hat{r}_i^\pm$ their positions (flux patches come in pairs in the absence of monopoles). The delta function is defined so that its integral over the unit sphere is unity. Standard results from potential theory allow the field at the Earth's surface to be written in the form

$$\mathbf{B}(\mathbf{r}) = \sum_i F_i [\mathbf{G}(\mathbf{r}, \hat{r}_i^+) - \mathbf{G}(\mathbf{r}, \hat{r}_i^-)] \quad (2)$$

where¹³

$$\mathbf{G}(\mathbf{r}, \hat{r}) = \nabla_r \left\{ \log \left[\frac{f+x-\mu}{1-\mu} \right] - \frac{2x}{f} \right\} \quad (3)$$

and $f^2 = (1 - 2x\mu + x^2)$, $x = c/r$ is the ratio of core to Earth radius, and μ is the cosine of the angular distance between $\mathbf{r}$ and $\hat{r}$. VGPs can be found from the vector field. By moving the flux points around and changing their strengths we can easily assess the effect of a changing core field on VGP path.

As examples, we give two extensions of the reversal model proposed by Gubbins⁸. The initial pattern of four patches, symmetrically placed about the equator near 65° N, S; 120° E, W⁴, is seen in the present geomagnetic field and is stable on the historical timescale; it has been proposed as a reflection of the underlying convective pattern¹⁴. The evolution is based on growth of pairs of flux patches in the Southern Hemisphere, presumed to result from flux expulsion, each about half the strength of the four main lobes, as envisaged previously⁸. In model 'E' one patch is carried west and then south to partially eliminate lobe 3, as is currently occurring beneath South

America, and the other patch is carried to the Pacific rim at 0° N; 90° W, where it remains (Fig. 1). Historical SV shows the development and drift of two such reverse flux patches. Here the process is repeated until all Southern Hemisphere flux is eliminated and then reversed. Recent SV gives no guide as to what then happens in the Northern Hemisphere, but we suppose that the flux is first eliminated and then reversed by northward migration of the equatorial patch. The positions of the flux points at each stage of the reversal are shown schematically in Fig. 2a.

The corresponding conventional VGP paths, recorded at a number of arbitrarily chosen sites, are shown in Fig. 3a; they are confined near longitude 90° E. It is an 'Asian path', but of course it would lie along the other half of the great circle had we used the same model for a R → N transition, giving an 'Americas path'. The dipole terms contribute only 34% of the surface energy $\oint \mathbf{B}^2 dS$ at the central configuration, which is surprisingly small considering the small longitude range of the VGP paths. The non-dipole terms give a site dependence to the VGP paths; they tend to move from east to west if the site is northeast or southwest of the point 0° N; 90° E and from west to east if the site is northwest or southeast of it.

A second sequence (model 'W', Fig. 2b) was generated by supposing that flux is reversed in the Northern Hemisphere first, which may occur if core flow carries flux across the Equator beneath the Atlantic (for which there is some evidence in core-field maps²) and eliminates lobe 1 either by carrying flux directly from 4 to 1, or by carrying one of a pair generated in the south Atlantic northwestwards to lobe 1, leaving the reversed patch on the Pacific rim. The VGP paths lie close to longitude 90° W (Fig. 2b); the VGP path lies in the Americas for a N → R transition, in Asia for a R → N transition.

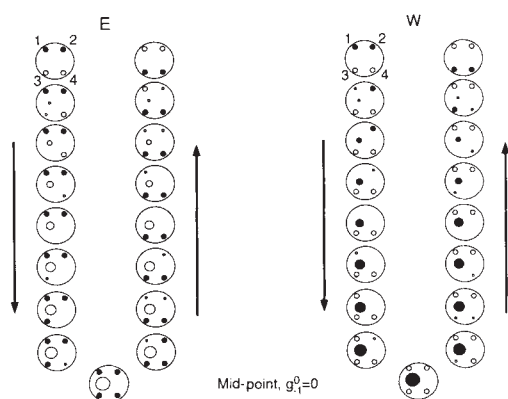


FIG. 2 a, Model E, a sequence of flux movements describing a reversal initiated in the Southern Hemisphere from flux patches originating in the Atlantic region (centre of the circle) and drifting west to accumulate at the Pacific rim and eliminate, then reverse, the initial configuration of four flux lobes. The flux patches are represented as points and the size of the dots represent their strength from 0.5 to 4 in steps of 0.5; black circles indicate negative flux and open circles positive flux. b, Model W, similar to model E but with flux eliminated from the Northern Hemisphere first.

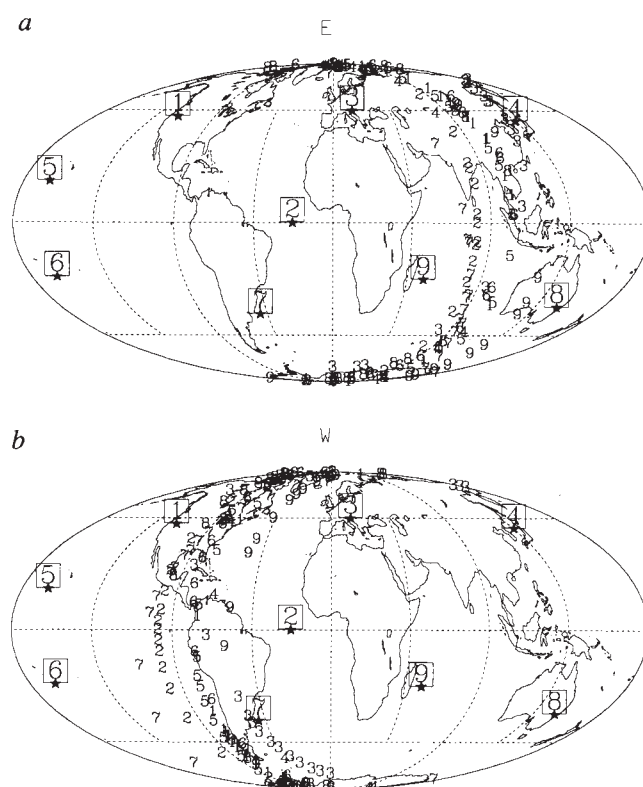


FIG. 3 a, VGP paths for nine numbered sites (chosen to be representative of real palaeomagnetic sites) for a normal-to-reverse transition. The paths all lie close to 90° E. b, VGP paths for model W. They all lie close to 90° W, opposite to model E. The difference arises because flux reversal initiates in the Southern Hemisphere in model E but in the Northern Hemisphere in model W. The longitude variation of VGP paths for both models is due in part to differences in the VGPs' starting longitude.

The intensity of the field is generally weak, falling to 10–20% of the initial strength over most of the Earth's surface, but in places it rises to high values. The weak intensity is caused by upward continuation through the mantle: the small-scale non-dipolar transition field is attenuated far more than the axial dipole. The occasional strong fields occur above the large flux patches, or where the effects of two or more patches reinforce. Transition fields are often thought of as weak but predominantly dipolar, as would occur if the dynamo were inactive and failing to regenerate the field. A strong, small-scale transition field could, however, correspond to particularly vigorous dynamo action.

These models are presented as examples of what can be done with simple equations. They show that the field can be strong at the core surface during transition, that longitude-confined VGP paths are consistent with strongly non-dipolar fields, and, most important, that blocking at the Pacific rim (as observed in the SV) can give rise to VGP paths through either of the two longitudes favoured by the data. The Asian path for N→R transitions results when the reversal initiates in the Southern Hemisphere; the Americas for the Northern Hemisphere. The model cannot explain N/R asymmetry nor the dominance of one half of the great circle over the other. Lavas appear to give

greater scatter than sediments (M. Prévot & P. Camps, manuscript in preparation), possibly indicating that sedimentary magnetization is smoothed. If the transition field fluctuates rapidly with a slight preference for the Pacific rim great circle, and there is some evidence from non-transitional fields for this¹⁶, the sediments may average out in preference for one of the two paths, explaining the extraordinary longitudinal confinement. □

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Plant-specific soil-borne diseases contribute to succession in foredune vegetation

W. H. Van der Putten, C. Van Dijk & B. A. M. Peters

Netherlands Institute of Ecology, Centre for Terrestrial Ecology,
PO Box 40, 6666 ZG Heteren, The Netherlands

ECOLOGICAL study of the role of soil microorganisms in vegetation succession has focused mainly on organisms affecting plant nutrition, such as mycorrhiza and nitrogen-fixing bacteria. But, soil-borne diseases are involved in the degeneration of *Ammophila arenaria* (Marram grass) and *Hippophaë rhamnoides* (Sea buckthorn), two plant species that dominate the coastal foredunes of Europe and are widely planted for sand stabilization. We have used reciprocal transplantation and report here that soil-borne diseases may contribute to the succession of foredune plant species. In pot experiments, plant species that succeed *A. arenaria* were tolerant of the soil-borne diseases of this species. Plant species that were grown in soils from both previous and later succession stages were reduced most in soils from the later stages. During foredune succession, therefore, plants disappear from sites where the soil has become colonized with specific growth-depressing microorganisms. The soil-borne diseases must have considerable importance for the outcome of interspecific competition and may be involved in patterns of clonal growth. The different sensitivities of plant species for the soil-borne pathogens could be an evolutionary response to selection pressures of the succession stage to which a species is confined by the combined effect of local abiotic and biotic environmental factors.

A characteristic of coastal foredune vegetation is the succession of zones dominated by single perennial plant species with clonal growth form. These plant species show roughly three phases of development: (1) colonization, (2) optimum growth and (3) degeneration and replacement by other plant species. Most explanations for these phases, particularly for degeneration, focus on changes in abiotic conditions^{1–5}, though some emphasize the ageing of the plants, especially their root systems^{6,7}. In north-western Europe, *A. arenaria* colonizes raised sites on the beach where it may survive considerable

burial by windblown sand^{8–11}. *A. arenaria* is succeeded by, among other species, *Festuca rubra* ssp. *arenaria*, and both benefit from burial by windblown sand^{6,10,12,13,14}; however the former is more tolerant of burial^{10,12}. The vigour of these plants declines if foredunes become stabilized and both species are replaced by *Carex arenaria*, *Elymus athericus* and *H. rhamnoides* ssp. *rhamnoides*, successively⁸.

Nematodes in the root zone of *H. rhamnoides* shrubs are involved in the degeneration of this species¹⁵. The nematodes *Tylenchorhynchus microphasmis* and *Londigorus dunensis*, when introduced into sterilized soil in which *H. rhamnoides* was grown, caused considerable growth reduction¹⁶. The discoloured, short-branched roots had disease symptoms similar to the roots of degenerated plants in the field¹⁶. But, only low densities of nematodes could be detected in the root zone of natural *Hippophaë* vegetation¹⁶. Soil-borne fungi were also implicated in the degeneration of the shrubs, and a synergistic interaction between nematodes and soil-borne fungi may increase their pathogenic activity^{17,18}. *H. rhamnoides* may not be the only foredune species that degenerates because of the activity of harmful soil-borne microorganisms¹⁹, and similar phenomena are involved in the degeneration of *A. arenaria* in stabilized dunes^{20–24}.

If soil-borne pathogens and parasites such as those involved in the degeneration of *A. arenaria* and *H. rhamnoides* are generally involved in foredune succession, then succeeding plant species must be tolerant of the soil-borne diseases of their preceding species. We investigated this hypothesis in two greenhouse experiments using seedlings of *A. arenaria*, *F. rubra* ssp. *arenaria*, *C. arenaria* and *E. athericus* grown reciprocally in sterilized and unsterilized soil samples collected from the root zone of natural monospecific stands. Chemical analyses of these soils indicated only very slight differences among soil origins (Table 1). Ample supply of nutrients²⁰ ensured the absence of possible side effects due to differences in initial soil fertility and nutrient flushes that may be induced by soil sterilization²⁵. The supply of nutrients might suppress the effect of vesicular-arbuscular mycorrhiza (VAM) and of free-living N₂-fixing bacteria that are active in dune soils^{26,27}. But previous experiments with *A. arenaria* indicate that growth in unsterilized soil from its root zone was reduced regardless of the quantity of Hoagland nutrient solution that was supplied²⁰. Reduced growth in the unsterilized sand samples in unfertilized soils was only enhanced

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TABLE 1 Chemical analyses of soils from the root zones of *Ammophila arenaria* (degenerated), *Festuca rubra* ssp. *arenaria*, *Carex arenaria* and *Elymus athericus*

Sampling site	pH KCl	Electro-conductivity ($\mu\text{S cm}^{-1}$)	Organic matter (%)	CaCO_3 (%)	N		Mg	K	Na	Cl
					Total	P				
					(mg per 100 g)	(mg per 100 g)	(mEquiv. per 100 g)	(mEquiv. per 100 g)	(mEquiv. per 100 g)	(mg per 100 g)
<i>A. arenaria</i> (degen.)	8.5	56	0.3	2.8	4.7	10.3	0.20	0.06	0.05	0.5
	0	0	0	0.2	1.5	0.6	0.006	0.006	0.01	0.2
<i>F. rubra</i>	8.5	82	0.3	3.7	5.7	12.0	0.24	0.13	0.09	2.8
	0.2	13	0.06	0.8	1.2	1.0	0.03	0.06	0.01	0.7
<i>C. arenaria</i>	8	71	0.6	5.4	18.7	13.3	0.22	0.08	0.05	0.6
	0.1	3	0.06	0.7	1.5	0.6	0.006	0.03	0.01	0.2
<i>E. athericus</i>	7.9	65	0.7	4.6	26.3	18.0	0.21	0.04	0.04	0.4
	0.06	3	0.1	0.9	2.1	1.0	0.02	0.01	0.01	0

Averages of three replicate samples are presented with, below, the standard deviation. After drying (35 °C) and sieving (2 mm), bulk soil samples were mechanically subdivided. Depending on analysis, either ground or unground subsamples were used. The pH of the soil was measured potentiometrically in 1:2.5 (w/v) suspensions of 1 M KCl. Carbonates were measured gas-volumetrically by treating samples with 4 M HCl. Organic matter was determined as weight loss after ignition at 430 °C for 24 h. Total N and P were measured colorimetrically in single soil digests³⁰. Exchangeable cations were determined by atomic absorption spectrophotometry after shaking soils with neutral ammonium acetate. Chloride and electrical conductivity analyses were done on 1:5 water extracts.

by the supplied nutrients, which demonstrated that the possible activity of beneficial soil organisms was dominated by that of harmful soil organisms²⁰.

In the first experiment, *A. arenaria* was grown in sterilized and in unsterilized beach sand, as well as in soil from the root zones of *A. arenaria* and of its successors, *F. rubra* ssp. *arenaria*, *C. arenaria* and *E. athericus*. Only in beach sand did sterilization not improve biomass production significantly. In all other unsterilized soils, productivity of *A. arenaria* was significantly lower than that in sterilized soil (Fig. 1). This suggests that in all soil origins except the beach sand, soil organisms were present that were harmful to *A. arenaria*.

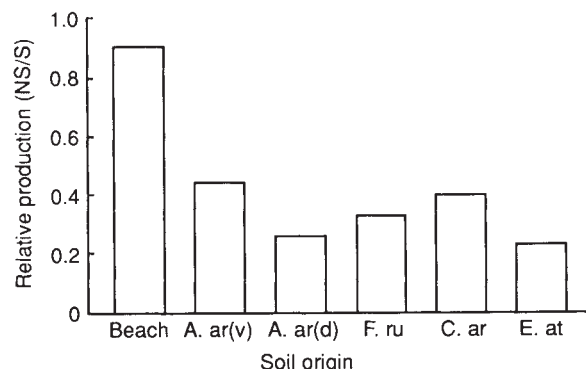
In the second experiment, *F. rubra* ssp. *arenaria*, *C. arenaria* and *E. athericus* were grown reciprocally in their own sterilized and unsterilized rhizosphere soils, as well as in soil from a degenerated stand of *A. arenaria*. In sterilized soils, productivity of each of the plant species was not significantly different (Table 2). In unsterilized soils, however, production of each of the plant species depended strongly on the origin of the rhizosphere soil. *F. rubra* ssp. *arenaria* showed a gradual decrease in biomass production along the series of unsterilized soil origins from *A. arenaria* to *E. athericus* (Table 2). In the rhizosphere soils from *C. arenaria* and *E. athericus*, biomass production of *F. rubra* ssp. *arenaria* was significantly increased by soil sterilization. *Carex arenaria* had a significantly lower production in both its

own unsterilized rhizosphere soil and that of *E. athericus* than it did in sand from *A. arenaria* and *F. rubra* ssp. *arenaria* locations (Table 2). The total biomass of *C. arenaria* was significantly increased by sterilizing the soil from the rhizosphere of *F. rubra* ssp. *arenaria*, *C. arenaria* and *E. athericus*. *E. athericus* had a significantly lower production in its own unsterilized rhizosphere soil than in unsterilized soil from *A. arenaria* and *F. rubra* ssp. *arenaria* sites (Table 2). Productivity of *E. athericus* was significantly increased by sterilizing soil from *C. arenaria* and *E. athericus* sites. When grown in unsterilized soil of their own origin as compared to sterilized soil, the relative production of *F. rubra* ssp. *arenaria* and *E. athericus* was significantly higher than that of *C. arenaria* (Fig. 2). *C. arenaria* barely survived in unsterilized sand from its own root zone, suggesting that the soil-borne pathosystem of this species is aggressive.

The general conclusion is that biomass production of the plant species examined was reduced by the soil-borne diseases of their own and of their successors. But there were differences among the plant species in unsterilized rhizosphere soil of the species preceding them. As *A. arenaria* is most vigorous when colonizing fresh windblown sand^{5,6,9,10,13,14}, it was suggested that this enables it to escape from harmful soil organisms^{23,24}. Nevertheless, as growth of *A. arenaria* has not been examined in the rhizosphere soil of the species usually preceding it, that is *Elymus farctus*, it cannot be verified whether *A. arenaria* is avoiding

FIG. 1 Production of total biomass of *Ammophila arenaria* grown in unsterilized soils compared to sterilized soils from: the beach, sites dominated by vigorous *A. arenaria* (A. ar(v)), degenerate *A. arenaria* (A. ar(d)), *Festuca rubra* ssp. *arenaria* (F. ru), *Carex arenaria* (C. ar) or *Elymus athericus* (E. at). Productivity was significantly increased by soil sterilization in all soil origins except beach sand.

METHODS. For each plant species, four random soil samples of 10 kg were collected from the upper 20 cm root layer of a monospecific stand at Voorne, The Netherlands (51.50°N, 4.05°E). The samples were mixed and sieved (10-mm mesh) and coarse fractions, including rhizomes and roots, were removed. The roots were chopped to 1-cm pieces and reintroduced into the sand and mixed gently. Half of each sample was sterilized by gamma-irradiation (4 Mrad; 1 rad=10 mGy). Tools and sieve were sterilized with alcohol (70%) after finishing each site. Six replicates were obtained by subdividing the sterilized and unsterilized bulk samples. Pots of 1.5 l were filled with 1,500 g of sand containing 10% moisture, based on dry weight. Tin foil lids protected the sand from desiccation. Uniform, 2-week-old seedlings of *A. arenaria*, obtained from seeds locally collected, were selected and planted in the pots (four per pot). The pots were placed in a greenhouse (average temperature 23 °C; minimum 18 °C and maximum 30 °C) and, when necessary, illumination (Philips HPIT 400 W, 4.8 W m⁻²) was supplied to keep the photoperiod at 12–14 h. Weekly, Hoagland nutrient solution³¹ was supplied and soil moisture was reset at 10% with demineralized water. Nutrients were increased each fortnight (12, 25 and 50 ml full-strength



Hoagland solution, respectively), to meet increasing plant requirements²⁰. After 6 weeks, plants were harvested and dry weights determined after drying for 48 h at 70 °C. Data of *A. arenaria* in soil from the beach, and sites with vigorous and degenerated *A. arenaria* are derived from ref. 24. Data were analysed statistically by two-way analysis of variance with factors being soil origin and soil sterilization. Tukey's multiple comparison was applied to compare treatment means as described in ref. 24.

TABLE 2 Total dry matter (mg per pot) of *Festuca rubra* ssp. *arenaria*, *Carex arenaria* and *Elymus athericus* grown in soils collected from their own and alien root zones

Plant species	Sand origin							
	<i>Ammophila arenaria</i> (degenerated)		<i>Festuca rubra</i>		<i>Carex arenaria</i>		<i>Elymus athericus</i>	
	NS	S	NS	S	NS	S	NS	S
<i>F. rubra</i> ssp. <i>rubra</i>	777 bcd	900 abc	613 cde	997 abc	404 de	1,370 a	218 e	1,128 ab
<i>C. arenaria</i>	714 bc	911 abc	679 c	977 ab	45.4 d	1,174 a	206 d	1,018 a
<i>E. athericus</i> *	1,060 cd	1,765 abc	1,326 bcd	1,511 abc	900 de	2,429 a	613 e	1,814 ab

The soils were either not sterilized (NS) or gamma-sterilized (S). Means of each plant species followed by the same letter do not differ at the $P < 0.05$. As in Fig. 1, except that six replicate samples were collected and half of each of the replicates was gamma-sterilized. Seedlings of *F. rubra* ssp. *arenaria*, *C. arenaria* and *E. athericus* were pregrown for 6 weeks in sterilized sand, after which plants of uniform size were selected and planted at a density of one per pot. The pots contained 1,350 g of sand containing 10% moisture, based on dry weight. The data per plant species were analysed statistically by two-way analysis of variance with factors soil origin and soil sterilization. As there was two-way interaction, treatment means were compared by Tukey's multiple comparison.

* Statistics applied after ln-transformation.

soil-borne diseases rather than tolerating those of its preceding species. Growth of *F. rubra* ssp. *rubra* was neither significantly reduced by the soil-borne organisms of the root zone of *A. arenaria* nor by those of its own. In contrast, growth of *C. arenaria* and *E. athericus* was significantly reduced in the rhizosphere soil of their immediate preceding species, as well as in their own rhizosphere soils. Nevertheless, growth of both *F. rubra* ssp. *arenaria* and *C. arenaria*, which has been examined in both rhizosphere soils of species preceding and succeeding them, was significantly less reduced in the soils of previous successional stages than in those of succeeding stages (Table 2). As the severity of the growth reduction in the different soils depended on the plant species examined, the soil-borne diseases seem to be plant-specific. Both *C. arenaria* and *E. athericus* have harmful organisms in their root zone just as *A. arenaria* and *H. rhamnoides* do. The occurrence of an aggressive soil-borne pathosystem in the soil of *C. arenaria* was concluded from severe growth reduction in its native soil. It may be that the typical clonal growth pattern^{3,4} reflects the occurrence of soil-borne diseases in the rhizosphere of *C. arenaria* and that the expansive horizontal growth³ is an effective mechanism in the escape from such aggressive pathocomplexes.

The biotic origin of the growth reduction in our experiments

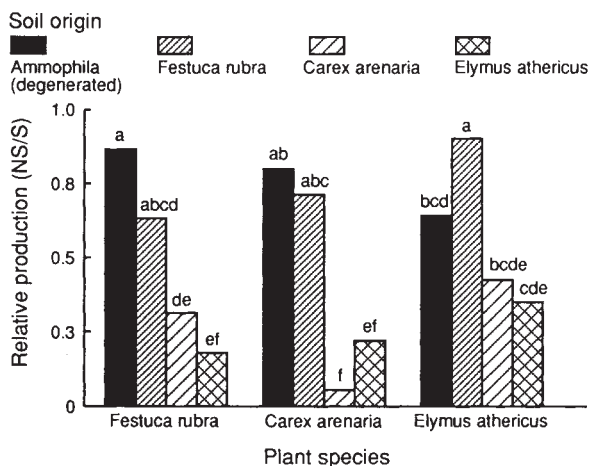


FIG. 2 Production of total biomass of *Festuca rubra* ssp. *arenaria*, *Carex arenaria* and *Elymus athericus* in unsterilized soil relative to that in sterilized soil. The soils originate from sites with pure stands of degenerated *Ammophila arenaria*, *F. rubra* ssp. *arenaria*, *C. arenaria* and *E. athericus*. METHODS. See Table 2. The relative productions were analysed by analysis of variance with factors being soil origin and plant species. As there was two-way interaction, Tukey's multiple comparison was applied to compare means of treatments and significant differences ($P < 0.05$) are indicated by different letters.

has thus been demonstrated. There were no significant differences in productivity among the sterilized soil samples, whereas large differences were found among the unsterilized rhizosphere soils. The increase of growth following soil sterilization does not indicate that allelopathy is a possible cause. In addition, the disease symptoms of the root systems, that is, brown-coloured, short-branched roots with few root hairs and lesions indicating fungal activity, and branching patterns showing activity of plant parasitic nematodes, make the involvement of allelopathy very unlikely. Although the individual components of soil-borne diseases can be identified, multiple synergistic relations among nematodes and soil-borne fungi^{16-19,22} lead to difficulties in determining which species are actually involved.

Natural succession of plant species has been attributed to the interaction of plant-specific characteristics and a wide range of abiotic and biotic environmental factors²⁸. The occurrence of plant-specific soil-borne disease complexes contribute a new and important factor. Interspecific competition as was shown for *F. rubra* ssp. *arenaria* and *A. arenaria*¹⁰ may be enforced, because in the natural substrate species to be replaced will be disfavoured by their own soil-borne pathosystem. This will supply succeeding species with a competitive advantage. In addition to suggestions that vesicular-arbuscular mycorrhiza (VAM) may increase uptake functions of the plants and reduce susceptibility of plants to soil pathogens²⁹, our previous experiments with rhizosphere soil of *A. arenaria* showed that the soil-borne pathogens may dominate the apparent positive effects of VAM and other beneficial microorganisms²⁰.

With respect to the role of soil-borne diseases, it has to be considered that the different sensitivities of plants could be a response to selection pressures of the successional stage to which a species is confined by other factors, such as salt spray, soil fertility or competition. Moreover, the importance of the soil-borne pathogens in the regulation of plant succession will depend on the integral effect of local abiotic and biotic environmental factors. To investigate possible influences of soil-borne diseases on vegetation, the spatial and temporal development of soil-borne pathosystems, the relation between soil-borne diseases and mosaic patterns in vegetation, as well as the relation between soil-borne organisms and natural selection in succession gradients, will be a great challenge for future ecological studies. □

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Floral mimicry by a plant pathogen

B. A. Roy

Botany Department, University of California, Davis, California 95616;
The Rancho Santa Ana Botanic Garden, Claremont, California 91711;
The Rocky Mountain Biological Laboratory, Gothic, Colorado 81224, USA

INSECTS can effect sexual reproduction in some plant pathogens, such as the rust fungi, by carrying spermatia (gametes) between different mating types^{1–5}. This function of insects is analogous to their role as pollinators of plants, and contrasts with their more widely known^{5–9} role as vectors of plant pathogens' infectious spores. Here I report an extraordinary case of pathogen-mediated floral mimicry that contributes to fungal reproduction. The rust fungus *Puccinia monoica* inhibits flowering in its host plants (*Arabis* species) and radically transforms host morphology, creating elevated clusters of infected leaves that mimic true flowers of unrelated species in shape, size, colour and nectar production. These fungal pseudoflowers attract insects which fertilize the rust. Because the pseudoflowers are highly successful in attracting pollinating insects, they may also affect the reproductive success of nearby flowering plants.

Species of *Arabis* (Brassicaceae), and a few other herbaceous mustards, become infected in late summer by wind-borne spores of the rust *Puccinia monoica* Arth.^{10,11}. Over the winter, rust mycelium invades the hosts' meristematic tissue, causing a systemic infection that affects all future growth. The rust fungus radically alters host morphology (Fig. 1). For example, infected plants of *A. holboellii* have twice as many leaves (41 ± 2.8 versus 21 ± 1.1 , $Z = 7.72_{115,116}$, $P < 0.0001$), twice as many leaf rosettes (3 ± 0.4 versus 1.5 ± 0.1 , $Z = 5.41_{116,116}$, $P < 0.0001$), and are twice as tall as non-flowering uninfected rosettes (97 ± 5.9 versus 46 ± 2.1 , $Z = 5.37_{74,91}$, $P < 0.0001$). Whereas uninfected plants form simple basal rosettes (Fig. 1a), infected plants form elongated stems crowned by dense, flower-like clusters of bright yellow infected leaves covered with a sticky, sweet-smelling exudate which is highly attractive to insects (Fig. 1c–e). Infected rosettes (pseudoflowers, Fig. 1c) bear little resemblance to uninfected rosettes (Fig. 1a), or to the flowers of host plants (Fig. 1b).

Pseudoflowers resemble unrelated flowers in form (Fig. 1e), colour (Figs 1e and 2), sweet-smelling odour, and sugar content (Table 2). Pseudoflowers often contain as much, or more, sugar than co-occurring flowers (Table 2) and other insect-pollinated flowers in similar habitats^{12,13}. The bright yellow surface of the infected petal-like leaves (Fig. 1c–e) is largely composed of spermatia filled with spermatia, receptive hyphae, and sugary spermatial fluid. The colour of pseudoflowers is indistinguishable in both the ultraviolet and visible spectra from that of yellow flowers of some co-occurring angiosperms and contrasts sharply with green vegetation (Fig. 2). Yellow is a particularly common colour for flowers of plants that, like *Arabis*, frequent high elevations, high latitudes¹⁴ and open habitats¹⁵.

Rust pseudoflowers attract a wide variety of flower-visiting insects including bees, butterflies, and flies (Fig. 1d, Table 2). Most rust fungi have outcrossing mating systems (are heterothallic) and require insect visitation for sexual reproduction^{1,2,5}. I used an insect exclusion experiment to test whether *P. monoica*, like other rusts, requires insect visitation for sexual reproduction (Table 1). Sexual spores (aeciospores) only formed on infected plants that were 'open-pollinated', or in cages that included insects. Infected plants from which insects were excluded remained yellow and continued to produce nectar, whereas those that were visited made sexual spores, turned green, and stopped producing nectar. Similar colour changes are often observed after pollination in some true flowers¹⁶.

Flies, which accounted for most of the visits to rust pseudoflowers at field sites in Colorado (Table 2), have been shown to be effective carriers of rust spermatia^{1,2,17} and are also important pollinators of montane and alpine flowers^{18,19}. Crab spiders and other predators of pollinators were also observed waiting for prey inside pseudoflowers, just as they do in flowers. The floral mimicry fools humans as well as insects: botany students at the Rocky Mountain Biological Laboratory have frequently collected pseudoflowers thinking they were flowers and, at a distance, many professional botanists have mistaken them for true flowers.

Insect visitation to fungal pseudoflowers accounts for a substantial fraction of pollinator activity in natural communities (Table 2). Flies, the most common visitors to both pseudoflowers and *Pulsatilla*, stayed 5 times longer per visit on pseudoflowers, with an average visit length of 102 seconds ($n = 46$) versus 21 seconds ($n = 43$) on *Pulsatilla*. Insect visitors also spent 88% of their time on pseudoflowers in mixed plots including *Claytonia lanceolata*, *Ranunculus inamoenus*, and *Mertensia fusiformis* (Table 2). Pseudoflowers may detain pollinators for relatively long visits because spermatial sugar is spread over the whole surface of the pseudoflower, rather than being concentrated in a nectary. Rust pseudoflowers that detain insects for long periods may promote their own fertilization. Insects can bring opposite mating types together without moving between pseudoflowers if both fungal mating types are found on the same host plant, as commonly occurs in multiple-spore infections by wind-borne pathogens^{2,20}.

By attracting pollinators, pseudoflowers may also affect the reproductive success of co-occurring flowering plants. Table 2 suggests that pseudoflowers could compete for pollinators in a way similar to that documented in some flower mixtures^{21–23} because insects may spend most of their time visiting pseudoflowers rather than true flowers. Alternatively, rust pseudoflowers could facilitate pollination of co-occurring flowers by increasing the total 'floral' display or by providing an additional food source. For example, when rust pseudoflowers and the buttercup *Ranunculus inamoenus* occur together (Fig. 1e), visitation to both the buttercups and pseudoflowers is increased (B.A.R., manuscript in preparation). The influence of pseudoflowers on pollination of adjacent plants will depend on several factors, including the relative density of infected *Arabis*, the extent to which pollinators are shared between pseudoflowers and flowers, and the breeding systems of those co-occurring

TABLE 1 Effect of insect visitation on rust fungus (*Puccinia monoica*) reproduction

Treatment	Pseudoflowers producing aeciospores	Pseudoflowers without aeciospores	N	Likelihood ratio G^*	P
Uncaged, 'open-pollinated'	20	0	20	53.59	<0.001
Caged, no pollinators	0	20	20	6.63	<0.01
Caged, 'dirty flies'	5	15	20	0.05	NS
Caged, 'clean flies'	3	17	20		
Overall test	28	52	80	63.74	<0.001

Puccinia monoica was unable to reproduce when insects were excluded. Not all infected plants that were visited by a single spore-covered fly produced spores, suggesting that: (1) the spermatia were incompatible, or (2) some caged flies did not visit the infected plant before they or the spermatia died. Some of the caged infected plants visited by 'clean' flies also produced spores suggesting that: (1) more than one mating type may be present on the same plant, or (2) the fungus is self-compatible but requires insect visitation to move spermatia between spermogonia, or (3) the putative 'clean' flies were also carrying spores. Infected plants in a meadow near the Rocky Mountain Biological Laboratory, Gunnison County, Colorado, USA, were tagged before the fungus became sexually receptive. Tagged plants were randomly assigned to four treatments: uncaged 'open-pollinated', caged (insects excluded), caged with 'dirty flies' (caged with one spermatia-covered fly captured feeding on an infected plant), or caged with 'clean flies' (caged with one fly captured on real flowers in an area where no rust infection was found within 1 km², and therefore presumed to be spermatia-free). The flies were introduced when the rust was sexually receptive; all flies died within 4 days. Cages consisted of chicken wire cylinders surrounded by Kleen Test plant sleeves; they excluded insects while allowing light, water and wind to pass through. Uninfected plants caged in this way grew and flowered normally, indicating that the cages did not affect plant growth.

* Multiple comparisons were corrected for experiment-wide error rate of 0.05, and the likelihood ratios were corrected with the Williams correction for small sample sizes²⁸.

plants (obligate outcrossers are most vulnerable to changes in pollinator behaviour).

The floral mimicry reported here is fundamentally different from previously reported pathogen effects on plant hosts. For example, although spermatial stages of some rust fungi produce sweet exudates and attract insects¹⁻⁵, the spermatia usually do not cover such large areas of the host, and the pathogen-mediated changes in host morphology do not resemble flowers.

Similarly, mummy-berry fungus has been reported to attract insects by secreting sugars and altering the ultraviolet reflectance of blueberry leaves⁷, but the insects disperse infectious spores rather than gametes, and infection does not radically alter host morphology. Finally, anther smuts invade host flowers, and disperse infectious spores by way of pollinators^{6,8,9}. By contrast, *Puccinia monoica* does not exploit its hosts' flowers; instead, it causes its host to construct a completely counterfeit flower, one

FIG. 1 Morphological transformation of *Arabis* by *Puccinia monoica* rust infection. *a*, Rosette stage of uninfected *A. holboellii*. Uninfected plants remain in the rosette stage for 2-5 years. *b*, Flowering stage of uninfected *A. holboellii*. Infection of this host almost always prevents flowering and is usually lethal; in 116 pairs of infected and uninfected plants, 71% of infected plants died without flowering (versus only 12% of the uninfected plants), and none of the infected plants set seed. *c*, Bolting stage of *A. holboellii* infected by *P. monoica*. Infected plants act like they are bolting (elongation of the stem before flowering), but elongation stops long before normal flowering height is attained and true flowering almost never occurs. *d*, *Polytonia zephyrus* (Nymphalidae) feeding on the spermatial fluid of *P. monoica* on *A. holboellii*. *e*, *A. drummondii* infected by *P. monoica* (right) strongly resembles *Ranunculus inamoenus* (left), in shape, size and colour in the visible spectrum.

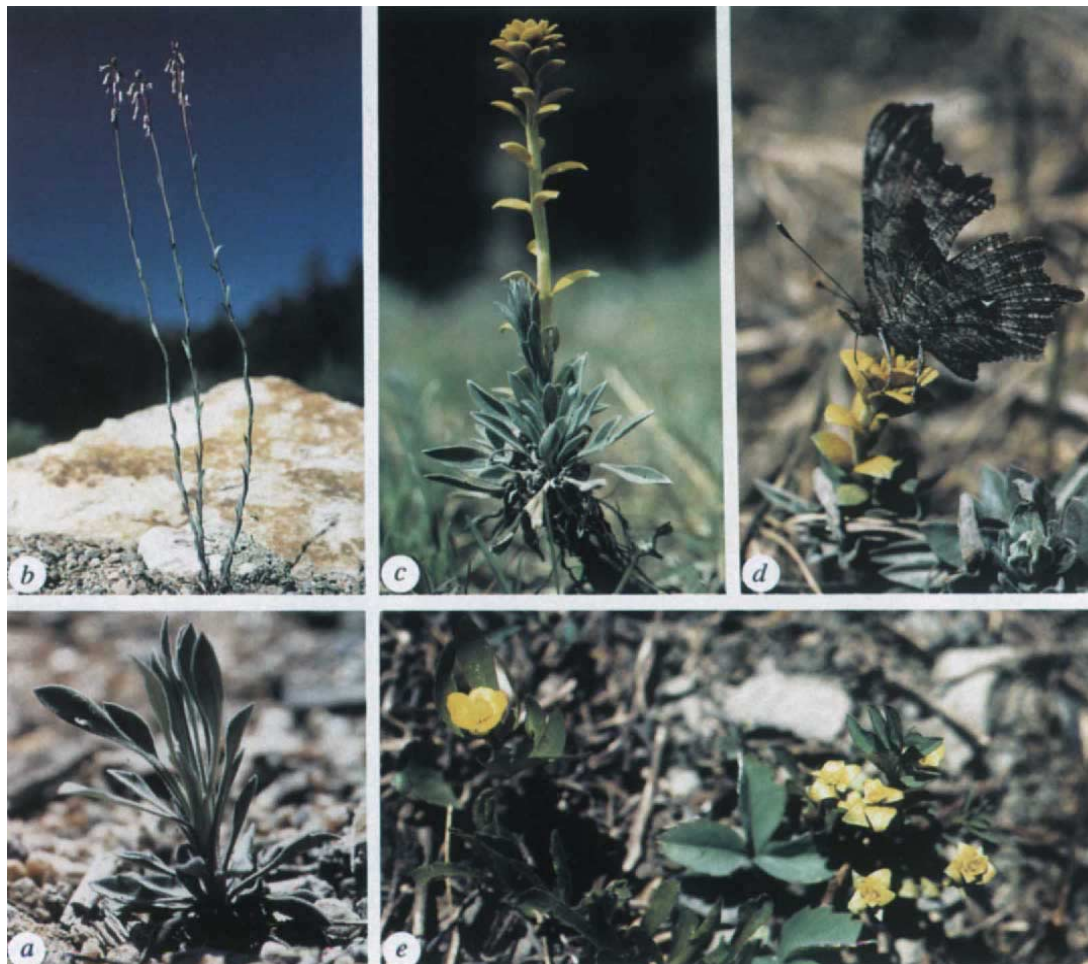


TABLE 2 Summary of flower sugar content and insect visitation to natural mixed plots of flowers including infected *Arabis* species

Plant species	Sugar per flower (mg)*†	Flowers in the plot (%)*	Visits to plots (%)	Time on plants (%)	Visits by flies (%)‡
Site one					
Rust pseudoflowers (on <i>A. holboellii</i>)	4.0 ± 0.70	52.0	45.1	72.2	100.0
<i>Pulsatilla patens</i>	0.12 ± 0.05	48.0	54.9	27.8	76.8
Site one total		n=98	n=102	108 min	n=89
Site two					
Rust pseudoflowers (on <i>A. drummondii</i>)	3.0 ± 0.32	34.2	37.0	87.9	90.0
<i>Claytonia lanceolata</i>	0.06 ± 0.01	2.3	0.9	0.03	33.3
<i>Mertensia fusiformis</i>	0.17 ± 0.02	47.1	9.9§	1.3	12.5
<i>Ranunculus inamoensis</i>	0.04 ± 0.01	16.3	52.2	10.7	14.8
Site two total		n=257	n=324	480 min	n=138

Insect observation took place between 11:00 a.m. and 3:00 p.m. during sunny, calm conditions. Site one consisted of 2.92 h of observation of three 1 m² plots near Almont in Gunnison County, Colorado (~2,700 m elevation). Site two consisted of 6.5 h of observation of three 1 by 2 m plots near Gothic, Gunnison County, Colorado (~2,957 m elevation). In all cases, insects were timed only when actually in the sexual parts of the flowers, or on the spermatial fluid of the rust fungus.

* Individual pseudoflowers were considered to be flower analogues because they are similar in shape and size to many co-occurring flowers (Fig. 1e). For example, the diameters of pseudoflowers and the diameters of flowers of *Ranunculus inamoensis* and *Claytonia lanceolata* differ only slightly: 10.67 ± 0.39 mm, versus 11.47 ± 0.21 mm and 14.87 ± 0.29 mm, respectively, n=40. Sugar content data are typically reported on a per-flower basis whether the flowers occur individually or in inflorescences^{12,13}. Here, single pseudoflowers are compared with single flowers. In the list above, only *Mertensia* has flowers in inflorescences.

† Sugar in spermatial fluid is fructose (identified chromatographically using Smith's solvent²⁹ of ethyl acetate, pyridine and water, and benzidine indicator reagent). Sugar content of 14 pseudoflowers per species was estimated by removing all infected leaves, soaking in distilled water for 1 h, and measuring sugar content of solution by refractometry. Sugar content of 20 flowers per species was estimated according to ref. 12; all sugar contents are reported as mean ± standard error, in sucrose equivalents.

‡ Fly visitors to infected *Arabis* include members of the Anthomyiidae, Bombyliidae, Calliphoridae, Muscidae, Stratiomyidae, Syrphidae and Tachinidae.

§ Actual insect visits to *Mertensia* could exceed observations; the inverted tubular flowers tended to hide visitors.

that mimics flowers of co-blooming species such as buttercups (Fig. 1e), rather than mimicking the host itself.

Rust fungi, including *Puccinia monoica*²⁴, are well known for their ability to affect the evolution of their host populations through selection on resistance alleles^{25–27}. My work indicates that some rust fungi, such as *P. monoica*, can also alter the growth and morphology of their hosts to a degree previously unknown. By redirecting host growth to produce strikingly flower-like forms, the fungus aids its own reproduction, alters the behaviour of insect pollinators, and possibly also affects the

reproductive success of co-occurring flowering plants. Thus this plant disease affects not only its hosts, but an entire natural community as well. □

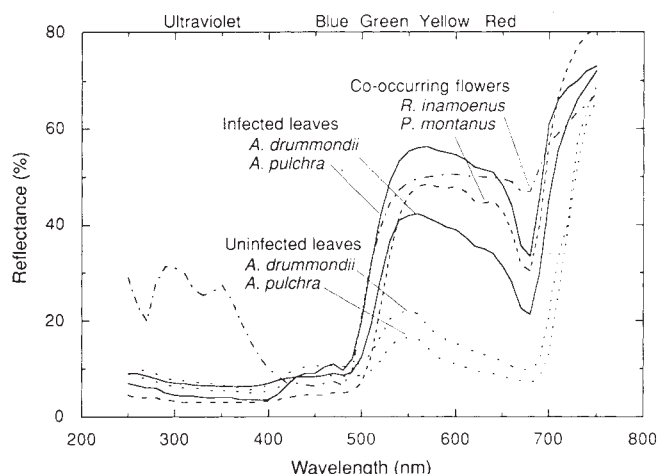


FIG. 2 Reflectance spectra of uninfected and infected *Arabis* leaves and petals of two co-occurring yellow flowers, *Ranunculus inamoensis* and *Pseudocymopterus montanus*. All spectra coincide in the visible spectrum; *Ranunculus inamoensis* also reflects in the near ultraviolet ('bee purple'). Leaves of *Arabis drummondii* were infected by *Puccinia monoica*, whereas leaves of *A. pulchra* were infected by *P. thlaspeos*. *P. thlaspeos* is very closely related to *P. monoica*³⁰ and also causes similar pseudoflowers to form on its hosts²⁴.

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Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis

Daniel R. Rosen*, **Teepu Siddique†**, **David Patterson‡**, **Denise A. Figlewicz§**, **Peter Sapp¶**, **Afif Hentati†**, **Deirdre Donaldson‡**, **Jun Goto§**, **Jeremiah P. O'Regan¶**, **Han-Xiang Deng†**, **Zohra Rahmani‡**, **Aldis Krizus§**, **Diane McKenna-Yasek***, **Annarueber Cayabyab†**, **Sandra M. Gaston¶**, **Ralph Berger‡**, **Rudolph E. Tanzi¶**, **John J. Halperin***, **Brian Herzfeldt†**, **Raymond Van den Bergh****, **Wu-Yen Hung†**, **Thomas Bird††**, **Gang Deng†**, **Donald W. Mulder‡‡**, **Celestine Smyth†**, **Nigel G. Laing§§**, **Edwin Soriano†**, **Margaret A. Pericak-Vance||**, **Jonathan Haines¶¶**, **Guy A. Rouleau§**, **James S. Gusella¶¶**, **H. Robert Horvitz||** & **Robert H. Brown Jr.*** #

* Day Neuromuscular Research Laboratory, Massachusetts General Hospital, Room 6627, MGH-East, Building 149, 13th Street, Charlestown, Massachusetts 02129, USA; † Department of Neurology, Northwestern University Medical School, Chicago, Illinois 60611, USA; ‡ Eleanor Roosevelt Institute for Cancer Research and the University of Colorado Health Science Center, Denver, Colorado 80206, USA; § Center for Research in Neuroscience, McGill University, and the Montreal General Hospital Research Institute, Montreal PQ H3G 1A4, Canada; ¶ Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, Boston, Massachusetts 02139, USA; ¶ Laboratory of Genetics and Aging, Neuroscience Center, Massachusetts General Hospital, Boston, Massachusetts 02129, USA; * Department of Neurology, Northshore University Hospital, Manhasset, New York 11030, USA; ** Department of Neurology, Universitaire Ziekenhuizen, Leuven 3000, Belgium; †† Department of Neurology, University of Washington School of Medicine, Seattle, Washington 98195, USA; ‡‡ Department of Neurology, Mayo Clinic, Rochester, Minnesota 55905, USA; §§ Australian Neuromuscular Research Institute, Nedlands, Western Australia, Australia; || Department of Medicine (Neurology), Duke University Medical Center, Durham, North Carolina 27710, USA; ¶¶ Molecular Neurogenetics Laboratory, Neuroscience Center, Massachusetts General Hospital, Boston, Massachusetts 02129, USA

AMYOTROPHIC lateral sclerosis (ALS) is a degenerative disorder of motor neurons in the cortex, brainstem and spinal cord^{1,2}. Its cause is unknown and it is uniformly fatal, typically within five years³. About 10% of cases are inherited as an autosomal dominant trait, with high penetrance after the sixth decade^{4,5}. In most instances, sporadic and autosomal dominant familial ALS (FALS) are clinically similar^{4,6,7}. We have previously shown that in some but not all FALS pedigrees the disease is linked to a genetic defect on chromosome 21q (refs 8, 9). Here we report tight genetic linkage between FALS and a gene that encodes a cytosolic, Cu/Zn-binding superoxide dismutase (SOD1), a homodimeric metalloenzyme that catalyzes the dismutation of the toxic superoxide anion O_2^- to O_2 and H_2O_2 (ref. 10). Given this linkage and the potential role of free radical toxicity in other neurodegenerative disorders¹¹, we investigated SOD1 as a candidate gene in FALS. We identified 11 different SOD1 missense mutations in 13 different FALS families.

We previously reported a CA dinucleotide repeat (D21S223) identified in cosmid 21-4.25 from the FALS-linked region¹². We have now found that exon 2 of SOD1 can be amplified by the polymerase chain reaction (PCR) from this cosmid (data not shown), indicating very close proximity of D21S223 and the SOD1 gene. We have confirmed the linkage of D21S223, and therefore SOD1, to FALS: D21S223 produces the highest lod scores yet detected by the Boston arm of the FALS collaborative

TABLE 1 Linkage analysis of FALS pedigrees with SOD1 marker D21S223 (DB1)

	Lod score							
	θ	0	5	10	15	20	25	30
All families*		$-\infty$	3.83	4.25	4.05	3.55	2.87	2.11
Ch. 21-linked families†		6.80	6.05	5.26	4.44	3.61	2.77	1.93

* Data are tabulated from an analysis of 12 families in the Boston arm of the FALS collaborative study^{8,12}.

† A subset of six families with a high (>80%) posterior probability of linkage to D21S223 as defined using the program HOMOG (ref. 14). Some of the 11 mutations described in Table 3 were detected in DNA from members of FALS families too small for significant linkage analysis. Such families could not be included in the linkage data summarized here.

study (Table 1). Using the program HOMOG^{13,14}, we have identified a subset of six of these FALS families in which the disease displays no recombination with D21S223 (Table 1; $z = 6.8$ at $\theta = 0$) and is likely to be tightly linked to SOD1; nine families of the Chicago group also display significant linkage to the SOD1 region of chromosome 21 (data not shown).

To determine whether FALS is associated with mutations in the SOD1 gene, we designed PCR primers for two of the five SOD1 exons based on the published sequence for human SOD1^{15,16}. These primers were used for PCR amplification of SOD1 exonic DNA from genomic DNA of normal, control individuals and of single FALS-affected individuals from families tightly linked either to SOD1 or neighbouring markers on chromosome 21q. The products of the PCR reactions were denatured and separated on a polyacrylamide gel (0.5×MDE, J. T. Baker) for single-strand conformational polymorphism (SSCP) analysis, which detects mobility shifts of single-strand DNA caused by sequence variations¹⁷. Autoradiograms of these gels revealed shifts in band mobility for 6 of the 15 families linked to the SOD1 region of chromosome 21q. Twelve of 135 families also revealed anomalous SSCPs; these families were too small for significant linkage analysis. Five of our FALS families are excluded from linkage to chromosome 21q (Table 2); none showed abnormal SSCPs. Representative SSCPs are shown in Fig. 1. No band shifts were detected in control DNA samples from normal individuals (140 and 112, respectively, for exons 2 and 4; Table 2).

SSCP analysis was then performed on all available DNA samples from members of families 3, 11 and 192C (data not shown). In each family, all affected individuals displayed the same band pattern as the originally characterized FALS patient.

TABLE 2 Eighteen single-strand conformational polymorphisms in exons 2 and 4 of SOD1 in FALS DNA*

	All FALS†	FALS-21‡	Control§
Exon 2 Normal	148	15	140
Variant	7¶	1	0*
Exon 4 Normal	150	15	112
Variant	11**	5	0

* See legend to Fig. 1 for methods.

† Familial ALS.

‡ Subset of families linked by HOMOG (ref. 14) to SOD1 (Boston) or adjacent markers (Chicago).

§ DNA samples from normal individuals unrelated to members of FALS families.

|| Six from Boston and nine from Chicago FALS pedigrees.

¶ Includes 4, 2 and 1 samples, respectively, from Boston, Chicago and Montreal FALS pedigrees.

* Twelve control DNA samples revealed weak and somewhat variable SSCPs; all were normal by sequence analysis.

** Includes 5, 5 and 1 samples, respectively, from Boston, Chicago and Montreal FALS pedigrees.

#* To whom correspondence should be addressed.

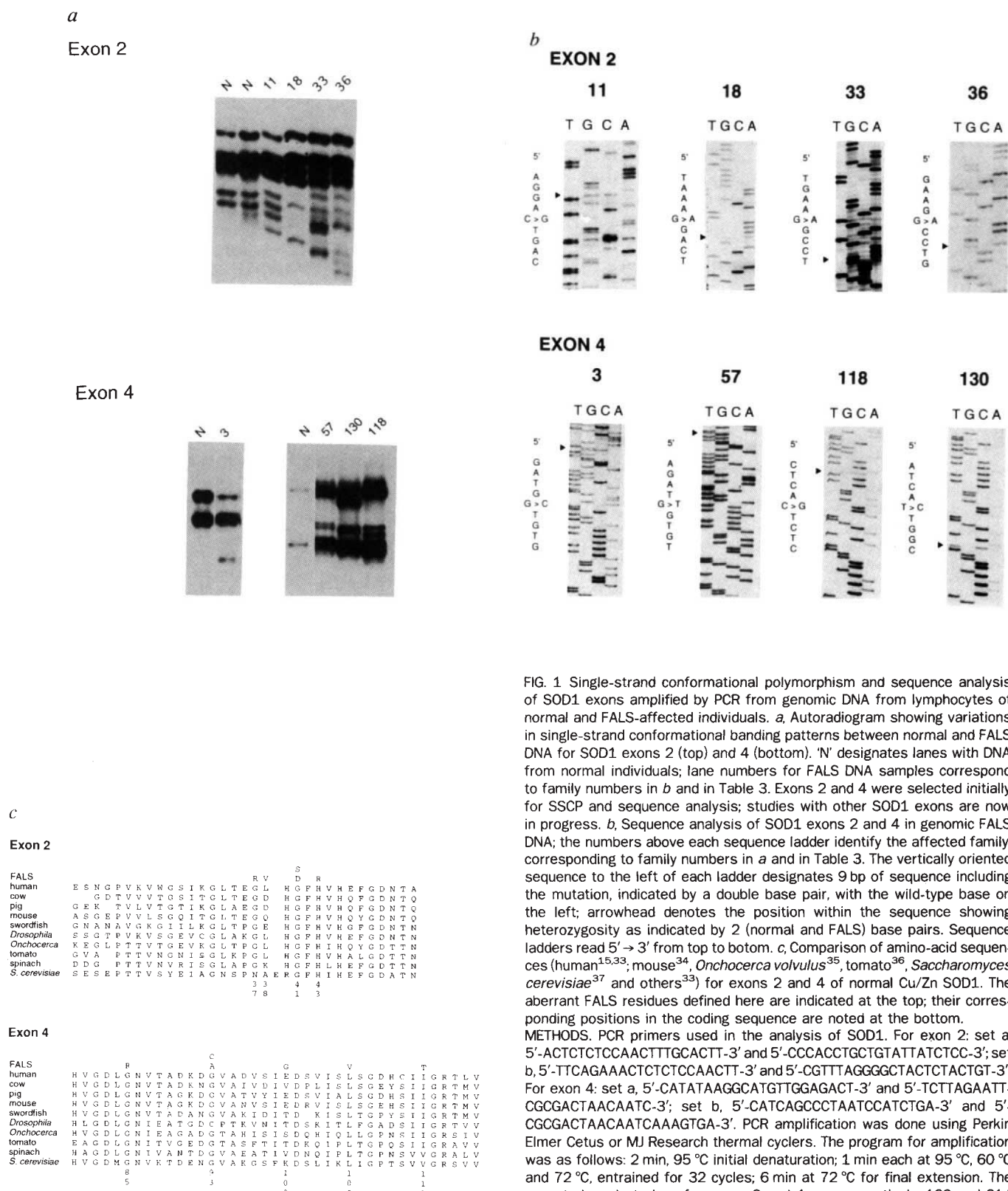


FIG. 1 Single-strand conformational polymorphism and sequence analysis of SOD1 exons amplified by PCR from genomic DNA from lymphocytes of normal and FALS-affected individuals. **a**, Autoradiogram showing variations in single-strand conformational banding patterns between normal and FALS DNA for SOD1 exons 2 (top) and 4 (bottom). 'N' designates lanes with DNA from normal individuals; lane numbers for FALS DNA samples correspond to family numbers in **b** and in Table 3. Exons 2 and 4 were selected initially for SSCP and sequence analysis; studies with other SOD1 exons are now in progress. **b**, Sequence analysis of SOD1 exons 2 and 4 in genomic FALS DNA; the numbers above each sequence ladder identify the affected family, corresponding to family numbers in **a** and in Table 3. The vertically oriented sequence to the left of each ladder designates 9 bp of sequence including the mutation, indicated by a double base pair, with the wild-type base on the left; arrowhead denotes the position within the sequence showing heterozygosity as indicated by 2 (normal and FALS) base pairs. Sequence ladders read 5' → 3' from top to bottom. **c**, Comparison of amino-acid sequences (human^{15,33}; mouse³⁴, *Onchocerca volvulus*³⁵, tomato³⁶, *Saccharomyces cerevisiae*³⁷ and others³³) for exons 2 and 4 of normal Cu/Zn SOD1. The aberrant FALS residues defined here are indicated at the top; their corresponding positions in the coding sequence are noted at the bottom. **METHODS**. PCR primers used in the analysis of SOD1. For exon 2: set a, 5'-ACTCTCTCCAACCTTGCACTT-3' and 5'-CCCACCTGCTGATTATCTCC-3'; set b, 5'-TTCAGAAACTCTCTCCAACCTT-3' and 5'-CGTTTAGGGGCTACTCTACTGT-3'. For exon 4: set a, 5'-CATATAAGGCATGTTGGAGACT-3' and 5'-TCTTAGAATT-CGCGACTAACAAATC-3'; set b, 5'-CATCAGCCCTAATCCATCTGA-3' and 5'-CGCGACTAACAAATCAAGTGA-3'. PCR amplification was done using Perkin Elmer Cetus or MJ Research thermal cyclers. The program for amplification was as follows: 2 min, 95 °C initial denaturation; 1 min each at 95 °C, 60 °C and 72 °C, entrained for 32 cycles; 6 min at 72 °C for final extension. The expected product sizes for exons 2 and 4 are, respectively, 132 and 214 bp for primer sets a, and 207 and 236 for primer sets b. SSCP analysis was done using MDE gels with the manufacturer's recommended protocol (J. T. Baker). Gels containing 5% glycerol were run at room temperature at 4 W for 16 h, then dried and exposed to film for autoradiography. Sequencing of PCR-amplified exon DNA was either by (1) purifying the resulting product on Centricon columns (Amicon) and sequencing directly with Sequenase kits (US Biochemicals); or (2) cloning the product into pUC19 from which the cloned inserts were used to verify the presence of the expected SSCPs and for sequencing with Sequenase kits. *Mae*III restriction enzyme digestion was as recommended (Boehringer-Mannheim).

TABLE 3 SOD1 mutations in familial amyotrophic lateral sclerosis

	Family	Base-pair change	Amino-acid change	Predicted restriction enzyme change	PCR product (bp)*	Restriction fragments (bp)	
						Normal	FALS-specific†
Exon 2	18, 594C¶	GGA → AGA	Gly 37 → Arg	+ <i>MaeIII</i> ‡§	132	132	72, 60
		CTG → GTG	Leu 38 → Val	– <i>HaeIII</i>	132	83, 49	132
		GGC → AGC	Gly 41 → Ser	– <i>StuI</i>	132	83, 49	132
				– <i>Eco57I</i>	132	97, 35	132
				– <i>HaeIII</i> ‡	132	83, 49	132
	36	GGC → GAC	Gly 41 → Asp	– <i>NlaIII</i>	132	87, 30, 9, 6	96
	220C	CAT → CGT	His 43 → Arg	+ <i>HinPI</i>	214	214	192, 22
	Exon 4	CGC → CGC	Gly 85 → Arg	+ <i>HhaI</i>	214	214	192, 22
				+ <i>FspI</i>	214	214	193, 21
					214		
	57	GGT → TGT	Gly 93 → Cys		214		
	3, 3-192C	GGT → GCT	Gly 93 → Ala	+ <i>SfaNI</i> ‡	214	112, 100	72, 40
	684C	GAA → GGA	Glu 100 → Gly	– <i>MboII</i>	214	88, 68, 58	156
	118	CTC → GTC	Leu 106 → Val	+ <i>DdeI</i> ‡§	214	120, 89, 5	83, 6
	130, 385C	ATT → AQT	Ile 113 → Thr	+ <i>BsrI</i> ‡§	214	124, 90	113, 11
				– <i>BslI</i> ‡	214	116, 98	214
				– <i>EaeI</i>	214	104, 57, 53	161

* Based on primer sets a (see Fig. 1 legend).

† Other size products are predicted but not listed because they are not FALS-specific.

‡ Predicted changes in the indicated restriction fragments have been confirmed in genomic DNA from FALS patients.

§ The FALS *MaeIII*, *DdeI* and *BsrI* fragments were not seen in DNA from 143, 73 and 73 control individuals, respectively.

|| All 73 DNA samples from normal controls showed the expected *HaeIII* restriction site.

¶ The suffix C denotes families analysed in Chicago. Families 3 and 3-192C are different branches of the same pedigree.

Additionally, other family members determined by haplotype analysis to be at risk for FALS had the distinctive SSCP variants, whereas several family members without the FALS haplotype did not. Thus, the FALS haplotype cosegregated with the SSCP variant.

We next performed direct sequencing of PCR-amplified DNA from exons 2 and 4 for 13 of the 18 FALS families with anomalous SSCP bands. In each instance, there was heterozygosity in the DNA sequence indicative of one normal and one abnormal chromosome. As summarized in Table 3, we identified single-base pair changes in all 13 of these families. These 13 mutations predict 11 distinct amino-acid substitutions. Two different amino-acid substitutions were detected in each of two codons (41 and 93; Table 3). In each of two codons (37 and 113), two apparently unrelated families have the same mutation. The same mutation in codon 93 was detected independently in two branches of the same family (designated 3 and 3-192C in Table 3).

Nine of the 11 sequence changes alter recognition sites for restriction enzymes (Table 3). For example, in family 11, a new *MaeIII* restriction enzyme digestion site (GTNAC) results from the C → G transition (normal sequence, CTGAC). To confirm these sequence changes, we digested PCR products of the corresponding exons with the appropriate restriction enzymes. *MaeIII* digestion of the exon-2 PCR product for affected members of family 11 produced three bands on a denaturing acrylamide gel (Sequagel 6; National Diagnostics): a 132-bp full-length product, and products of 72 and 60 bp, which are the fragment sizes expected, given the extra *MaeIII* site in the mutant DNA. These fragments were not detected in normal, control DNA samples from 70 unrelated members of our ALS families or from 73 unrelated members of reference pedigrees in the Centre d'Etude du Polymorphisme Humain (CEPH¹⁸). The base-pair changes in families 3, 118, 130 and 684C introduce other novel restriction sites; additionally, in families 33, 36, 130 and 684C the single-base changes eliminate restriction sites normally present in SOD1.

These studies identify 11 single amino-acid changes in SOD1 on the basis of the genomic DNA sequences of members of 13 different FALS families. These changes were not detected in more than 100 chromosomes from normal individuals and so

appear not to be simply normal allelic variants. Instead, these mutations occur in association with FALS. Although it is conceivable that the mutation that causes FALS leads to the accumulation of mutations in other genes, including SOD1, this possibility seems unlikely. We believe the simplest hypothesis is that the mutations we have identified in the SOD1 gene cause FALS.

These observations raise several questions. First, how could mutations in SOD1 underlie the pathogenesis of familial ALS? SOD catalyses the conversion of the toxic superoxide anion radical O_2^- to hydrogen peroxide, which can then be converted to water by glutathione peroxidase or catalase¹⁰. By reducing superoxide levels, SOD also prevents the reaction of superoxide with nitric oxide (NO) to form peroxynitrate anion ($ONOO^-$), which can in turn generate the even more toxic hydroxyl radical HO^* ¹⁹. Reactive free radicals such as superoxide and OH^* can be directly toxic to cells²⁰; SOD-defective mutants of bacteria, yeast and the fruitfly *Drosophila melanogaster* are all hypersensitive to oxidative damage^{21–24}. Furthermore, free radicals have been proposed to cause neuronal injury in several neurological disorders, including Parkinson's disease and ischaemic brain injury¹¹. Thus, one possibility is that in FALS patients the activity of SOD1 is reduced, leading to an accumulation of the toxic superoxide radical. Alternatively, the activity of SOD1 might be increased, leading to excessive levels of hydrogen peroxide and the highly toxic hydroxyl radical, which can be formed through the reaction of hydrogen peroxide with a transition metal such as iron²⁵.

Why a SOD1 abnormality might selectively damage motor neurons is unclear, given that this gene is broadly expressed²⁶. Nonetheless, overexpression of SOD1 in transgenic mice using normal SOD1 regulatory sequences led to an apparently specific defect in distal motor neuron terminals of the tongue and hindlimbs^{27–29}, indicating that this gene can selectively affect motor neurons. By contrast, patients with either complete or partial monosomy 21 for the region of chromosome 21 that includes the SOD1 locus have severe multisystem pathology^{30–32}.

How might the 11 apparent missense mutations in SOD1 affect its activity, and how might they account for the autosomal dominant inheritance of FALS? Most of the changes we observed affect SOD1 residues that are highly conserved among

organisms^{15,33-37} (Fig. 1c) which suggests that these sites are important for SOD1 function. One possibility is that these mutations decrease or eliminate SOD1 activity. But most loss-of-function mutations cause a recessive rather than a dominant effect^{38,39}. Exceptions can arise in proliferating cells that allow a somatic mutation to cause a loss of function of the second allele of the gene, as in the case of retinoblastoma⁴⁰; this situation seems unlikely to arise in a nonreplicating cell such as the motor neuron.

Rather than decreasing SOD1 activity, putative FALS mutations might either increase SOD1 activity or have a dominant-negative effect⁴¹, such that the mutant SOD1 protein is not only functionally defective but also inhibits the function of the normal SOD1 protein expressed from the normal allele. Consistent with the first hypothesis, both Ile 113 and Leu 106 are thought to be involved in forming intrachain hydrogen bonds important for the increased thermostability of a mutant form of SOD1 (ref. 42); it is plausible that SOD1 proteins with amino-acid changes at these residues are of increased stability and hence of increased activity. Consistent with the second hypothesis, one of the sites abnormal in FALS patients, Ile 113, has been implicated in intrachain hydrogen-bond formation between SOD1 monomers^{43,44}; perhaps the normal and mutant proteins combine to form an inactive heterodimer.

The human genome encodes two other SOD proteins besides the cytosolic Cu/Zn SOD1 protein: the mitochondrial Mn-dependent SOD2 (ref. 45) and the extracellular (EC) SOD3 (ref. 46). We are currently exploring the possibility that mutations in the genes encoding Mn SOD2 or EC-SOD3 are responsible for cases of FALS not linked to the Cu/Zn SOD1 region of chromosome 21 (ref. 8) or for other neurodegenerative disorders.

Perhaps the most important implication of our finding of various SOD1 mutations in FALS patients is its potential therapeutic benefit. If indeed toxicity caused by oxygen free radicals is the primary pathogenetic mechanism for motor neuron death in FALS and perhaps in sporadic ALS as well, measures that diminish this toxicity might blunt the devastating course of this disease. Clinical trials of either SOD itself or compounds that penetrate the central nervous system and decrease levels of free radicals should be feasible; indeed, such drugs are currently being tested for treatment of Parkinsonism⁴⁷. It might be particularly attractive to consider similar studies in individuals with defined SOD1 mutations. □

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Cytokine regulation of neuronal differentiation of hippocampal progenitor cells

Mark F. Mehler*†, Renato Rozental*†, Maryjane Dougherty*, David C. Spray† & John A. Kessler*†

Departments of *Neurology and †Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461, USA

‡Institute of Biophysics 'Carlos Chagas Filho', Federal University of Rio de Janeiro, RJ 21941, Brazil

THE signalling mechanisms governing haematolymphopoiesis and those regulating neural development may be closely related, as indicated by similarities of higher-order structure and function of the cytokines involved¹, of the regional and temporal regulation of their transcription and translation²⁻⁶, and of their bioactivity⁷⁻¹⁰. Here we investigate this possible evolutionary connection using retroviral transduction of a temperature-sensitive mutant form of the SV40 large T antigen to develop conditionally immortalized murine embryonic hippocampal progenitor cell lines¹¹⁻¹⁴. Treatment of these cells with cytokines that are thought to participate in progressive lymphoid maturation, immunoglobulin synthesis¹⁵⁻¹⁸ and erythropoiesis^{19,20} causes progressive neuronal differentiation, as defined by morphological criteria, successive expression of increasingly mature neurofilament proteins²¹⁻²³, and the generation of inward currents and action potentials. The cytokine interleukin(IL)-11 induces expression of action potentials that are insensitive to tetrodotoxin, which is indicative of developmentally immature sodium channels²⁴. By contrast, for expression of more mature action potentials²⁴ (tetrodotoxin-sensitive) one of the interleukins IL-5, IL-7 or IL-9 must be applied in association with transforming growth factor- α after pretreatment with basic fibroblast growth factor. Our results suggest that the mechanisms regulating lineage commitment and cellular differentiation in the neural and haematopoietic systems are similar. Further, they define an *in vitro* model system that may facilitate molecular analysis of graded stages of mammalian neuronal differentiation.

A retroviral construct¹⁴ containing a temperature-sensitive form of the SV40 large T antigen and a dominant selectable

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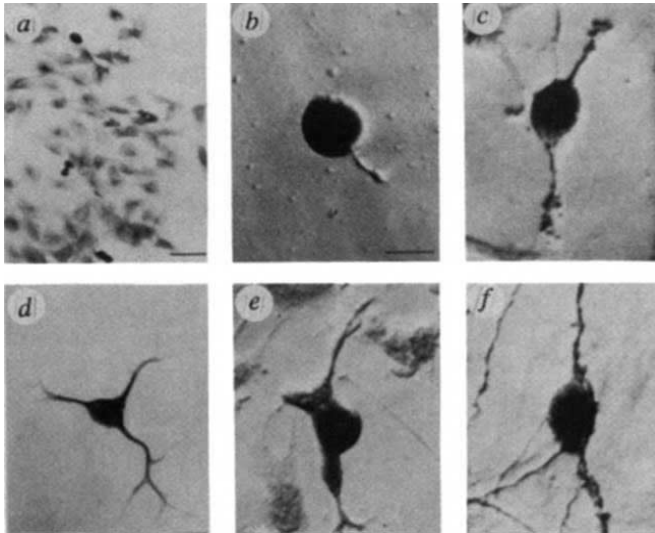


FIG. 1 Morphological and immunocytochemical characteristics of neuronal progenitor cells (MK31) exposed to various growth factors. *a*, Plated in FCS at 33 °C and immunostained with a polyclonal antibody for nestin. *b*, As *a*, but high-power view similarly immunostained for nestin. *c*, SFM³⁰ at 39 °C, immunostained for NF66. *d*, As *c*, but with interleukin-11 as well for six days, immunostained for NFH-P. *e*, As *c*, but with IL-7 and TGF- α for six days after one day's pretreatment with bFGF; immunostained as in *d*. *f*, Primary dissociated hippocampal neurons (E17) propagated for six days and immunostained as in *d*. Scale bars: *a*, 50 μ m; *d*, 20 μ m; *b*, *c*, *e* and *f*, 10 μ m. METHODS. Murine hippocampi from embryonic day 17 were enzymatically dissociated and plated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37 °C. Two hours later medium was replaced by supernatant derived from two ψ 2 NIH3T3 producer lines containing replication-defective retrovirus carrying the *neo* gene and either tsA58 or U19tsa temperature-sensitive alleles of the SV40 large T antigen. The supernatant was titred to cause successful retroviral transduction of 1–5 cells per 35 mm² dish in 3 h in the presence of 8 μ g ml⁻¹ hexadimethrine bromide. After infection, virus-containing medium was replaced by fresh medium and the cultures maintained at 33 °C; 48 h later, 400 μ g ml⁻¹ G418 was added and subsequently maintained for selection. Microscopic G418-resistant colonies containing 20–40 cells were picked with cloning rings 3 to 13 weeks later and serially expanded. For immunocytochemistry, cells were fixed in cold absolute methanol (10 min) or 4% (w/v) paraformaldehyde in 0.1 M phosphate (pH 7.4), blocked with 5% normal goat serum (30 min) and incubated with primary antibody at 4 °C (24 h), biotinylated secondary antibody (30 min), and vectastain kit ABC reagent (1 h). Immunoreactivity was revealed with diaminobenzidine (0.5 mg ml⁻¹, with 0.01% peroxide in 50 mM Tris, 0.14 M NaCl, pH 7.4).

marker, the neomycin-resistance gene, was used to immortalize neural stem and progenitor cells from dissociated embryonic day-17 mouse hippocampus^{11–14}. Microscopic colonies of resistant cells under selection were isolated in the presence of 10% fetal calf serum (FCS) at the temperature (33 °C) permissive for T-antigen expression. These evolving neural cell lines were initially comprised of flat, polygonal cells that lacked immunocytochemical reactivity for antigens expressed in neural precursor cells or terminally committed progeny. However, over hours to days, a subpopulation of more rounded cells developed (Fig. 1*a*) which was initially widely dispersed within this stromal population, showed rapid proliferation, extended minimal neurites (Fig. 1*b*) and immunoreactivity for the class VI intermediate-filament protein nestin²⁵, which is indicative of a neuronal precursor. When the cell lines were switched to the temperature not permissive for T-antigen expression (39 °C) in serum-free medium, the growth rate dramatically slowed and the rounded, nestin-positive cells began to extend longer neuritic processes and develop immunoreactivity for an early neurofilament protein, NF66 (ref. 21; Fig. 1*c* and Table 1). To assess the feasibility of promoting neurogenesis *in vitro*, we analysed responses of our neural lines to a new set of growth factors (Tables 1 and 2). After preliminary characterization of cytokine and neurotrophin effects on morphology and neurofilament protein expression^{21–23}, we concentrated on one hippocampal cell line (MK31) which gave the most pronounced response. All growth factors were studied singly or in combination with basic fibroblast growth factor (bFGF) and transforming growth factor- α (TGF- α), chosen because of their neural bioactivity and expression in the developing brain^{26–28}.

In serum-containing medium at 33 °C, 6.6% of MK31 cells were immunoreactive towards nestin and assumed a rounded morphology without exhibiting neuronal characteristics (Table 1). At 39 °C in serum-free medium, 17.5% of cells were rounded and were now immunoreactive to the early-neurofilament protein NF66, but showed little evidence of morphological maturation (Fig. 1*c*; Table 1). Neuronal differentiation, as evidenced by morphological and immunocytochemical criteria, was fostered by a few cytokines (IL-5, -7, -9 and -11), either alone or in combination with TGF- α (Table 1). Individual cytokine concentrations were optimized to induce maximal neuronal differentiation (Table 1). Each of these culture conditions enhanced neurite outgrowth and promoted nuclear enlargement

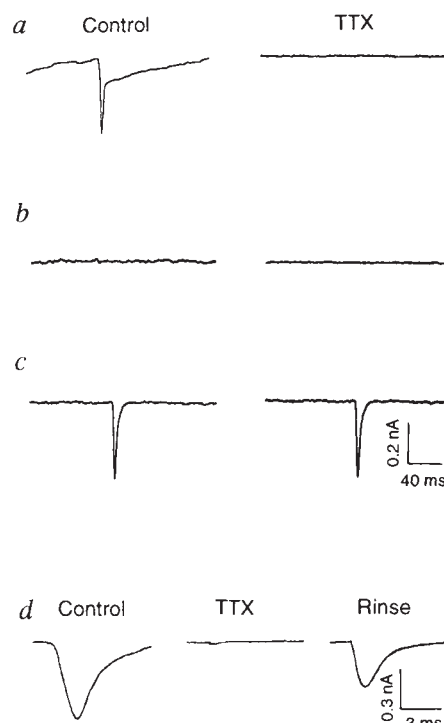
TABLE 1 Morphological and immunocytochemical indices of progressive neuronal maturation

	N	Per cent IF ⁺ cells	IF protein	Morphological maturation (%)
Untreated				
FCS/33 °C	20	6.6 $\pm$ 0.6	Nestin	0
SFM/39 °C	19	17.5 $\pm$ 1.0	NF66	1.8 $\pm$ 0.4
bFGF, then TGF- α	11	29.6 $\pm$ 2.0	NFH-P	5.6 $\pm$ 1.0
IL-7	9	24.9 $\pm$ 0.8	NFH-P	21.4 $\pm$ 1.3
IL-11	15	23.9 $\pm$ 0.8	NFH-P	27.7 $\pm$ 0.9
bFGF, then IL with TGF- α :				
IL-5	7	21.3 $\pm$ 2.5	NFH-P	38.0 $\pm$ 2.9
IL-7	8	26.0 $\pm$ 3.9	NFH-P	39.4 $\pm$ 2.9
IL-9	7	36.2 $\pm$ 3.9	NFH-P	57.9 $\pm$ 2.8
IL-11	8	21.9 $\pm$ 1.3	NFH-P	26.9 $\pm$ 1.2

The percentage of IF⁺ cells ($\pm$ s.e.; N gives the number of experiments) that express intermediate filament (IF) proteins is shown. NF66, low-molecular-weight neurofilament protein; NFH-P, high-molecular-weight phosphorylated neurofilament protein. For each experimental condition, the most mature developmental marker expressed in the sequence nestin-NF66-NFH-P is listed. The rightmost column represents the percentage of IF⁺ cells satisfying the morphological criteria for progressive neuronal maturation (neurite extension, presence of growth cones, nuclear maturation, non-neuronal cellular process outgrowth). Cells (40,000 per cm²) were plated onto 35-mm² dishes and evaluated on culture day 4 by counting 0.5 cm² in each of four quadrants. Total cell counts and viability were verified in parallel plates by resuspension and counting with a haemocytometer using trypan-blue exclusion; this was replicated a minimum of seven times for each group. Growth factor concentrations: bFGF and TGF- α , 5 ng ml⁻¹; IL-7, 10 U ml⁻¹; IL-5, -9, 40 U ml⁻¹; IL-11, 50 U ml⁻¹. Individual cytokine concentrations were chosen from an expanded log range centred on values that optimized progressive haematopoietic maturation. The following growth factors either alone or in various combinations were ineffective for neuronal precursor cell differentiation: interleukins-1, -2, -3, -4, -6, -8 and -10; colony-stimulating factors (CSF) macrophage-CSF, granulocyte-CSF and granulocyte/macrophage-CSF; ciliary neurotrophic factor, leukaemia inhibitory factor, oncostatin-M; stem cell factor, erythropoietin; transforming growth factor- β 1, epidermal growth factor, platelet-derived growth factor-AA, platelet-derived growth factor-BB, tumour necrosis factor- α ; nerve growth factor, brain-derived neurotrophic factor, neurotrophin 3. FCS, fetal calf serum; SFM, serum-free medium.

FIG. 2 Electrophysiological characteristics of cultured cells. *a*, Murine embryonic day-17 hippocampal neurons maintained in SFM for six days; *b*, conditionally immortalized neuronal precursor cells in SFM at 39° in the absence or presence of assorted growth factors (Tables 1 and 2); *c*, as *b*, but with IL-11 as well for six days; *d*, as *b*, with IL-7 and TGF- α , preceded by one day bFGF pretreatment. Note scale difference in this panel.

METHODS. Electrophysiological analysis of cells cultured for 4 to 19 days was performed in constantly flowing external solution using current or voltage clamp recording techniques. External solution (in mM): NaCl 165, KCl 5, CaCl₂ 2, HEPES 5, D-glucose 10 (pH 7.3; 340 mOsm). Inward currents and action potentials were recorded under whole-cell configuration using an LM-EPC 7 (Medical Systems, NY) patch-clamp system (21–22 °C). All records were filtered at 3 kHz cut-off (Bessel filter) and stored onto video tape (Neurocorder, model DR-484, Neuro Data Instruments). Currents and action potentials were analysed using the pCLAMP program. High-resistance seals to cell surfaces were obtained by applying suction to the rear of polished pipettes (resistance 2–5 M Ω), filled with (in mM): CsCl 80, CsF 80, Cs-EGTA 10, HEPES 10 (pH 7.3; 340 mOsm); access to cell interiors was achieved with brief light suction. Holding potentials were in the range –65 to –75 mV. Excitability was observed as spontaneous activity (see *a* for example), activity evoked by long depolarization by 20–30 mV (*b*, *c*), or as inward currents recorded during brief 20-mV depolarizing voltage steps (*d*). Records were not corrected for leakage currents and pipette series resistance compensation was rarely used. To evaluate sensitivity to tetrodotoxin (TTX), 0.3 μ M TTX was added to the external solution for 5 min. When TTX sensitivity was observed, cells were rinsed with normal saline to determine recovery in *d*; panel labelled 'Rinse' displays currents recorded 7.5 min after the reintroduction of normal solution.



and complexity. Moreover, each promoted cellular expression of the phosphorylated form of the high-molecular-weight (M_r 200K) mature neurofilament protein (NFH-P), with reduced expression of nestin and NF66 (Fig. 1*d*, *e*). When one of the interleukins IL-5, -7 or -9 was coupled with TGF- α after pretreatment with bFGF, cellular forms showed even more pronounced neuronal morphological maturation (Fig. 1*e*; Table 1). These cells (representing between 21% and 36% of total cells; Table 1) continued to express NFH-P.

The hallmark of the neuronal phenotype is cellular excitability. We therefore decided to characterize further the developmental phenotype of MK31 under conditions favouring neuronal maturation by making whole-cell voltage-clamp recordings on 172 cells maintained for 4–19 days (Table 2). Inward currents and action potentials were preferentially assessed from cells that were morphologically like neurons (with neurite outgrowth, cellular polarity, enlarged and complex nucleus, and growth cones). Electrophysiological responses were all maximal by six days in culture. There were no inward currents after treatment with a range of growth factors, whether added singly, concurrently or sequentially ($n = 44$; Table 1). In contrast, treatment with interleukins-7 or -11 induced expression of inward currents in response to depolarizing steps and propagation of action potentials that were tetrodotoxin-insensitive, indicative of sodium channels with developmentally 'immature' physiological properties²⁴ (Tables 1 and 2; Fig. 2*c*). These responses occurred in 10% and 26%, respectively, of 38 surveyed neuronal forms. But neuronal species treated with IL-5, -7 or -9 and TGF- α , after treatment with bFGF, gave action potentials sensitive to tetrodotoxin, which is consistently shown in primary hippocampal cultures (Figs 1*f*, 2*a*) and is indicative of sodium channels with more mature physiological properties (Fig. 2*d*; Table 2). In contrast, application of IL-11 and TGF- α after bFGF treatment did not result in further morphological or electrophysiological maturation beyond that with IL-11 alone (Tables 1 and 2). Electrical excitability was never seen in untreated cultures (31 cells) or in cultures pretreated with bFGF followed by TGF- α (12 cells).

These observations suggest that a selective hierarchy of growth factors is crucial for progressive neuronal differentiation in the

mammalian brain. Ligands of two distinct classes of growth factor receptors, the tyrosine kinase and the cytokine receptor superfamilies¹⁸, are instrumental in these developmental events, suggesting that complex and synergistic interactions are occurring within developmentally regulated signal transduction pathways^{18,29}. These findings demonstrate biological activity in a model of mammalian neurogenesis of four interleukins, three of which (IL-5, -7 and -9) have previously been implicated as late-acting, synergistic factors in progressive stages of haematolymphoid maturation^{15–20,29}, and of TGF- α , which is abundant in developing brain²⁶. In contrast, IL-11 has synergistic interactions with early stage-specific cytokines IL-3, IL-4 and

TABLE 2 Electrophysiological analysis of the effects of growth factor treatment on neuronal maturation

Growth factor(s)	N	Excitable cells (%)	Tetrodotoxin sensitivity
Untreated	31	0	—
bFGF, then TGF- α	12	0	—
IL-7	19	10	—
IL-11	19	26*	—
bFGF, then IL with TGF- α :			
IL-5	8	38†	+
IL-7	10	20‡	+
IL-9	13	46§	+
IL-11	9	11	—
combined ILs	40	30	—
Primary neuronal cultures (untreated)	7	100	+

Excitability was assessed on cells treated for 4 to 19 days with each agent under voltage- or current clamp conditions. Cells were considered excitable if they showed spontaneous action potential activity or if regenerative activity or inward currents were evoked by depolarizations. Examples of excitable and inexcitable cells are shown in Fig. 1. Experimental protocols were identical for electrophysiological studies of neuronal maturation for additional growth factors ($N = 44$; Table 1).

* $P = 0.0055$; † $P = 0.0061$; ‡ $P = 0.055$; § $P = 0.00024$; || $P = 0.0071$ (Fisher's exact test for unpaired data was used to evaluate the significance of the differences between various treated and the untreated group).

granulocyte/macrophage-colony stimulating factor (GM-CSF) to shorten the G0 period of haematopoietic stem cells^{1,24}. Further, it is intriguing that IL-11, which in our system induced intermediate stages of neuronal maturation, also functions at several stages of erythropoiesis¹⁹; this haematopoietic sub-lineage is selectively affected in homozygous mice deficient for the retinoblastoma protein Rb, whose only other developmental defects are restricted to intermediate stages of neurogenesis⁴⁻⁶. Our results should facilitate analysis of mechanisms governing neurogenesis both *in vitro* and *in vivo* after implantation of developmental stage-specific neural progenitor cells. □

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Deregulation of *Pax-2* expression in transgenic mice generates severe kidney abnormalities

Gregory R. Dressler, J. Erby Wilkinson*,
Uwe W. Rothenpieler, Larry T. Patterson,
Lisa Williams-Simons & Heiner Westphal

Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, Bethesda, Maryland 20892, USA

* Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee 37901, USA

THE *Pax* genes comprise a family of transcription factors active in specific tissues during embryonic development and are associated with at least three developmental mutations in mouse and man^{1,2}. In the developing kidney, *Pax-2* is expressed in the induced mesenchyme, in the ureter epithelium, and in early epithelial structures derived from the mesenchyme³. *Pax-2* expression is repressed upon terminal differentiation of the renal tubule epithelium, but persists in the undifferentiated epithelium of human Wilms' tumours^{4,5}. We have produced a dominant gain-of-function mutation in transgenic mice by deregulating the expression of the mouse *Pax-2* gene. The data obtained with four independently derived transgenic embryos and with one transgenic line demonstrate that deregulated *Pax-2* expression results in histologically abnormal and dysfunctional renal epithelium with properties similar to congenital nephrotic syndrome. Thus, repression of *Pax-2* is required for normal kidney development and persistent expression of *Pax-2* may restrict the differentiation potential of renal epithelial cells.

The mammalian kidney is induced by a reciprocal interaction between the ureter epithelium and the metanephric mesenchyme, both derived from intermediate mesoderm cells⁶. This inductive interaction initiates a sequence of morphogenetic changes that begins with the aggregation and polarization of the mesenchyme and leads to the formation of mature renal tubules, glomeruli and collecting ducts. Although the nature of the inductive signals has been extensively characterized, the subsequent genetic changes in response to such signals and the genes that control cell differentiation and cell lineage

TABLE 1 Transgenic mice with deregulated *Pax-2* expression

Experiment	Births	Transgenic mice	Comments
1 CMV-Pax2b	41	1 (C2425)	Apparently normal, all transgenic offspring exhibit open eyes, kidney defects and die shortly after birth
2 CMV-Pax2b	44 (E18)	4	All 4 had open eyes and kidney defects

TABLE 2 Serum analysis of normal and transgenic newborns

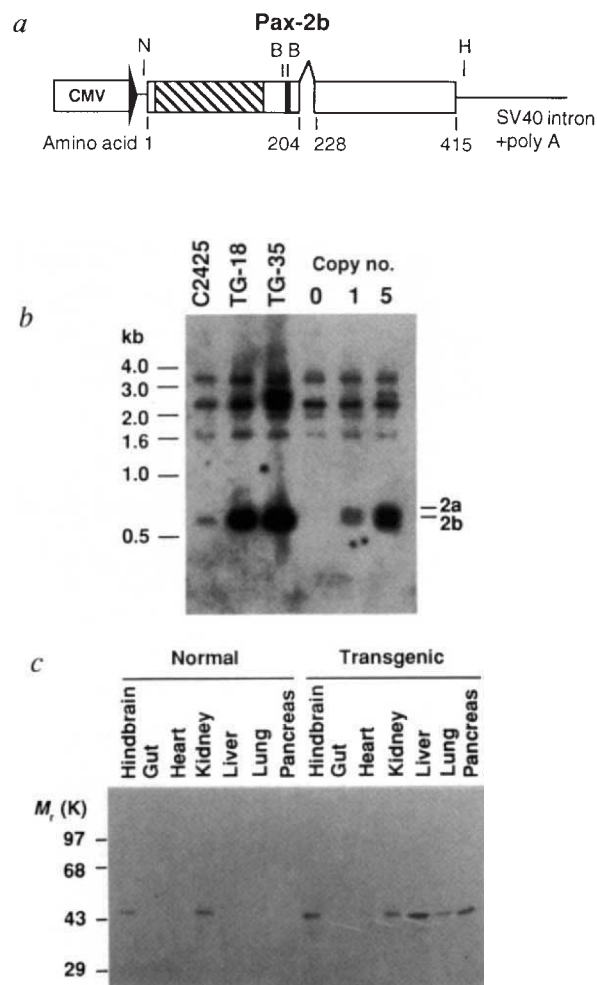
Test	Normal*	Transgenic*
BUN (mg dl ⁻¹)	23 ($\pm$ 7.0), <i>n</i> =14	42 ($\pm$ 19.3), <i>n</i> =14
K ⁺ (mEq l ⁻¹)	5.9 ($\pm$ 0.36), <i>n</i> =12	7.0 ($\pm$ 0.29), <i>n</i> =12

* Standard deviation given in parentheses.

specification in the developing kidney remain to be determined. A good candidate for a regulator of early kidney development is the *Pax-2* gene³. Because of the precise down regulation of *Pax-2* expression during renal epithelium maturation and the persistent expression of *Pax-2* in the undifferentiated epithelium of Wilms' tumours^{4,5}, we postulated that persistent *Pax-2* expression could lead to developmental abnormalities. To test this hypothesis, *Pax-2* was deregulated in transgenic mice.

The human cytomegalovirus (CMV) immediate early gene promoter^{7,8} was fused to a complementary DNA encoding the more abundant 392-amino-acid Pax-2b form^{3,4} (Fig. 1a) and injected into zygotes (Table 1). Initial attempts yielded only one apparently normal transgenic male (C2425; Fig. 1b). However, approximately 25% of C2425 offspring contained the transgene, had open eyes, and died 1-3 days post partum. Litter sizes were normal and no evidence of resorption was seen at late developmental stages. The transgenic pups were able to suck and had no overt neurological abnormalities. The low transgene copy number of C2425 (Fig. 1b), the less than 50% transmission rate, and the founder's survival, suggests mosaicism for the CMV-Pax2b transgene. Given the post-natal lethality, zygote injection was resumed and the fosters were killed 18 days after embryo transfer (E18). Four additional transgenic embryos were identified at E18 and all had precocious opening of the eyes. Endogenous *Pax-2* can be detected in the normal kidney and

FIG. 1 *a*, Construction of Pax-2b transgene. The Pax-2 sequences were derived from the cDNA clones p31A and pCPX³. The CMV promoter was cloned as a 550-bp *Xba*I/*Hind*III fragment from the vector CMV-CAT^{7,8}. The 3' untranslated sequences, the SV40 early region intron, and the polyadenylation site were derived from the vector RSV-CAT^{1,3}. *b*, Genomic Southern blot analysis of transgenic mouse DNAs and control DNAs. C2425 is a founder male, TG-18 and TG-35 are independently derived embryos taken at 18 days gestation. The controls are 0, 1, or 5 genome equivalents of plasmid used for injection diluted in normal mouse DNA. 5 µg of DNA was cut with *Bam*HI and *Hind*III, run on a 1.2% agarose gel, blotted and hybridized using standard methods¹⁴. The probe was a 570-bp *Bam*HI/*Eco*RI fragment from clone c31A (ref. 3). The transgenic mice were generated as described^{15,16}. *c*, Pax-2 protein expression in normal and transgenic newborn tissues. The specificity of the Pax-2 antibodies and the methods used have been described⁴. Equivalent amounts of protein were loaded in each lane as judged by Coomassie blue staining of duplicate gels (data not shown).



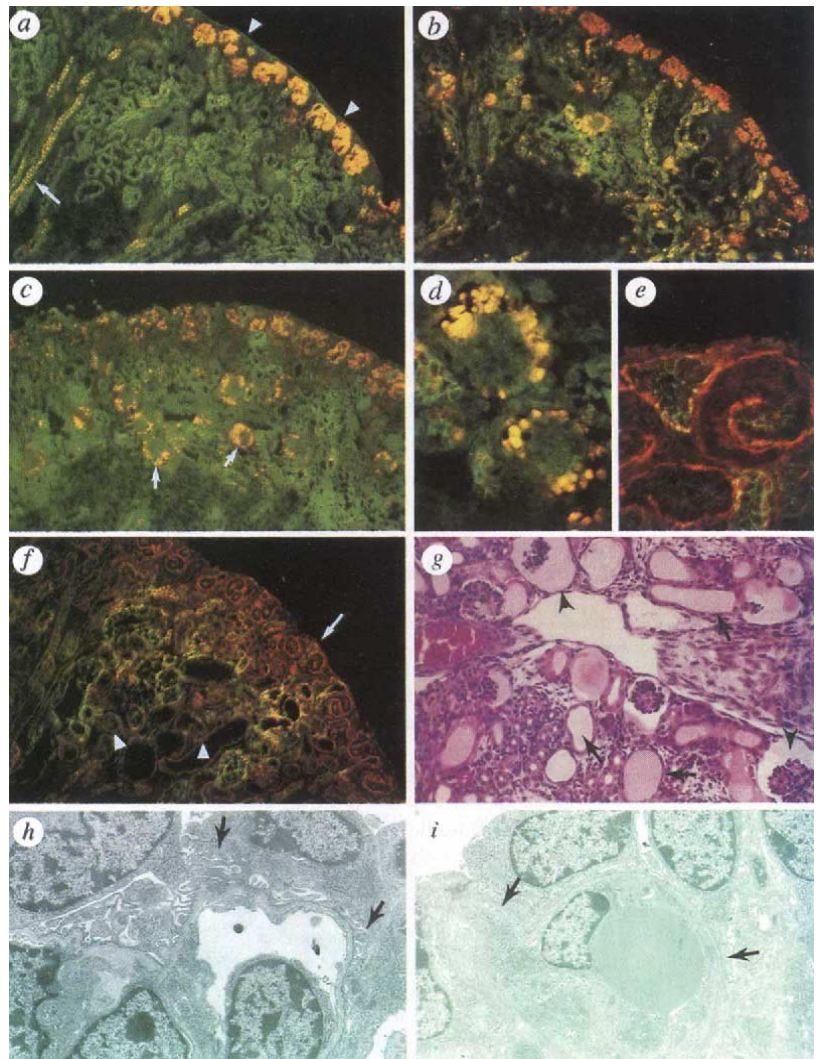
hindbrain (Fig. 1c), consistent with previous findings^{4,9,10}. However, the transgenic newborns also had Pax-2b protein in the heart, liver, lung, pancreas and low levels in the gut.

The kidneys from transgenic newborns (C2425) and from four independently derived E18 transgenic embryos were sectioned and stained with antibodies against Pax-2 and uromodulin, an epithelial specific adhesion molecule¹¹. The normal Pax-2 expression pattern shows staining in the nephrogenic zone and in the collecting ducts (Fig. 2a). Transgenic kidneys were markedly different in that Pax-2 expression was found in large clusters of cells throughout the cortex, in addition to the nephrogenic zone (Fig. 2b, c). Ectopic Pax-2 expression was highest in the semi-circular cluster of the visceral glomerular epithelium, the prospective podocyte cells (Fig. 2d). The control kidneys have a dense tubular organization as visualized with the uromodulin antibody, that stains all proximal, distal and ductal epithelium (Fig. 2a). In transgenic kidneys, uromodulin staining was normal in the developing epithelium at the periphery but was reduced and more diffuse in the mature tubules (Fig. 2b, c). Kidneys taken from four independently derived C2425 transgenic embryos showed a similar Pax-2 expression pattern (data not shown). Antibodies against laminin were used to stain the developing basement membranes of the renal epithelium. Transgenic kidneys showed normal basement membrane formation in the comma and s-shaped bodies at the periphery (Fig. 2e) but the more mature tubules, that have become dilated, exhibit very diffuse staining (Fig. 2f). Laminin staining in glomerular epithelium appeared normal (data not shown). Six different C2425 transgenic newborns and six non-transgenic littermates were fixed and processed for histo-

pathology and ultrastructural analysis. The brain, liver, heart, lung, spleen, skeletal muscle, gonads, and digestive tract of the transgenic mice showed no gross histological abnormalities. The lens and retina were normal despite precocious opening of the eyes. In the kidneys, however, the Pax-2b transgenics exhibited moderate, multifocal microcystic tubular dilation (Fig. 2g) with protein-filled lumens. Bowman's spaces were also frequently filled with protein and the associated glomeruli were atrophic. Lesions were limited to the most mature nephrons. Ultrastructural analysis revealed a severe abnormality in the capillary loops characterized by a paucity of podocyte foot processes and poorly developed endothelial fenestrae (Fig. 2h, i). Glomerular basement membranes were normal. By electron microscopy, the tubular changes were most prominent in the proximal tubules but occasional distal tubules were also involved. Dilated proximal tubules were lined by flattened epithelial cells with attenuated brush borders. The cytosol of some proximal tubular epithelial cells was expanded by protein-filled inclusions.

Transgenic and normal littermates from C2425 matings were analyzed for renal function at 1–2 days post partum (Table 2). A twofold increase in blood urea nitrogen BUN levels was observed in the transgenic mice. Potassium levels were unusually high in transgenic newborns, with an average of 7.0 mEq l⁻¹, compared with 5.9 mEq l⁻¹ in the control animals. Serum sodium and chloride levels were normal in the transgenic mice. Proteinuria approaching 300 mg dl⁻¹ was also detected exclusively in the transgenic newborns, consistent with the histological findings. The data indicate severely compromised renal function as a probable cause of death in newborns. Furthermore, the development of

FIG. 2 Immunostaining and histopathology of normal and transgenic kidneys. Immunostaining in *a-d* shows Pax-2 in red and uvomorulin in green. Immunostaining in *e* and *f* show laminin in red and uvomorulin in green. *a*, Normal kidney at 1 day post partum. Note the restriction of Pax-2 to the peripheral nephrogenic zone (arrowheads) and collecting ducts (arrow). The renal tubules are discernible by uvomorulin staining. *b*, Transgenic C2425 offspring at 1 day post partum. Note expression in cell clusters throughout the cortex and diffuse uvomorulin staining. *c*, Different C2425-derived newborn, showing cortical Pax-2 expression in cells of the presumptive visceral glomerular epithelium (arrows). *d*, Higher magnification of *c*, showing glomerulus. *e*, Laminin and uvomorulin expression at the periphery of transgenic kidney. Note characteristic comma-shaped body. *f*, Laminin and uvomorulin expression is normal at periphery (arrow), but decreases and becomes more diffuse in mature tubules (arrowheads). *g*, High magnification of cortex from transgenic 1-day-old kidney. Note multifocal microcysts (arrows) and protein filled Bowman's spaces (arrowheads). *h*, Electron micrograph of normal visceral glomerular epithelium. Note foot processes of podocyte cell (arrows). *i*, Transgenic visceral glomerular epithelium showing absence of foot processes at the podocyte cell membrane (arrows). METHODS. Sections were cut on a cryostat, then fixed for 5 min in 3% paraformaldehyde for Pax-2 staining, or for 10 min in methanol at -20°C for laminin staining. Immunostaining was as described⁴. The laminin polyclonal antibodies recognize the B1 and B2 chains and were a gift from H. Kleinman. Preimmune IgG antibodies did not show any reactivity with sections from normal or transgenic mice (data not shown). Micrographs were taken with a Nikon Microphot-FXA and 35 mm film, with a total magnification of $\times 32$ (*a*, *b*, *c*, *f*) and $\times 64$ (*d*, *e*). For histology and electron microscopy, magnification is $\times 75$ (*g*), $\times 7,500$ (*h*) and $\times 6,500$ (*i*).



microcysts, foot process loss, and proteinuria is reminiscent of congenital nephrotic syndrome¹². Glomerular sclerosis is not evident in the mice, although this may be due to the early death of the animals.

Our data demonstrate that persistent expression of *Pax-2* is not compatible with normal kidney development. *Pax-2* expression is activated in the induced mesenchyme and may function in the transition of mesenchyme to epithelium by modulating specific genes required for these early events. However, *Pax-2* is normally repressed upon maturation of the mesenchyme-derived renal epithelium. In the CMV-*Pax2b* transgenic kidneys, the early events of nephrogenesis are not affected and the newly formed, but still not fully differentiated, epithelium appears normal. It is precisely in the mature renal tubules and glomeruli that the effect of persistent *Pax-2* expression is most striking. The data suggest that *Pax-2* can instill a non-terminally differentiated state upon cells and thereby block the normal molecular genetic pathways that determine the eventual cell fate. We have not observed gross abnormalities in other tissues, including the neural tube where endogenous *Pax-2* expression is evident^{9,10}. However, more subtle neural phenotypes may go undetected given the perinatal lethality and the lack of specific markers for neural subtypes in the spinal cord. It is certainly possible that particular kidney cells express co-factors required for *Pax-2* function and thus are more affected by *Pax-2* overexpression. In any event, the deregulated expression of *Pax-2* in transgenic mice has a

clear detrimental impact on development and may thus help to define the roles of Pax genes in normal development and oncogenesis. □

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Selection for T-cell receptor $V\beta$ - $D\beta$ - $J\beta$ gene rearrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis

Jorge R. Oksenberg*, Michael A. Panzara*, Ann B. Begovich†, Dennis Mitchell*, Henry A. Erlich†, Ronald S. Murray‡, Richard Shimonkevitz‡, Martina Sherritt§, Jonathan Rothbard||, Claude C. A. Bernard§ & Lawrence Steinman*

* Department of Neurology and Neurological Science, Stanford University School of Medicine, Stanford, California 94305-5235, USA

† Department of Human Genetics, Roche Molecular Systems, Emeryville, California 94608, USA

‡ Rocky Mountain MS Center, Englewood, Colorado 80150, USA

§ Laboratory of Neuroimmunology, LaTrobe University, Victoria 3083, Australia

|| ImmuLogic Pharmaceutical Corporation, Palo Alto, California 94304, USA

MULTIPLE sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system in which a restricted cellular immune response has been observed. In order to establish whether such T cell responses are likely to be antigen-specific particularly with regard to myelin basic protein (MBP), we analysed T-cell receptor (TCR) gene rearrangements directly from MS brain plaques, using the polymerase chain reaction on reverse transcribed messenger RNA, and compared these with TCR of previously described MBP-specific T cell clones from MS and the rat model experimental allergic encephalomyelitis. Rearranged $V\beta 5.2$ genes were detected in the brains of all patients who were HLA DRB1*1501, DQA1*0102, DQB1*0602, DPB1*0401. The $V\beta 5.2$ - $D\beta$ - $J\beta$ sequences in these MS brain plaques revealed five motifs. One of the common motifs was identical to that described for the VDJ region of a $V\beta 5.2$ T-cell clone. This clone was from an MS patient who was HLA DRB1*1501, DQB1*0602, DPB1*0401, and it was cytotoxic towards targets containing the MBP peptide 89–106 (ref. 1). The deduced amino-acid sequence of this VDJ rearrangement, Leu-Arg-Gly, has also been described in rat T cells cloned from experimental allergic encephalomyelitis lesions, which are specific for MBP peptide 87–99 (ref. 2). VDJ sequences with specificity for this MBP epitope constitute a large fraction (40%) of the TCR $V\beta 5.2$ N(D)N rearrangements in MS lesions. The capacity of rat T cells with these VDJ sequences to cause experimental allergic encephalomyelitis² and the prevalence of such sequences in demyelinated human lesions indicate that T cells with this rearranged TCR may be critical in MS.

TCR $V\alpha$ - $J\alpha$ - $C\alpha$ and $V\beta$ - $D\beta$ - $J\beta$ - $C\beta$ rearrangements were identified in MS lesions using amplification of reverse-transcribed mRNA by polymerase chain reaction (PCR) with one of 18 $V\alpha$ - or one of 21 $V\beta$ -specific oligonucleotide primers^{3,4}. All specimens were typed for the HLA class II loci DRB1, DQA1, DQB1 and DPB1 (refs 5–9). Eight of sixteen MS patients were DRB1*1501, DQA1*0102, DQB1*0602 and either DPB1*0401 or 0402, here referred to as molecular phenotype '1'. This haplotype, which corresponds to the cellular type HLA-DR2,Dw2, is associated with increased susceptibility to MS in certain caucasoid populations^{10,11}. MS patients with phenotype '1' had an increased frequency of certain $V\alpha$ and $V\beta$ rearrangements.

Given the increased frequency of $V\beta 5$ rearrangements in HLA DR2-restricted T cell clones reactive to MBP^{1,12}, we asked whether $V\beta 5$ was transcribed more frequently in MS brains of patients with phenotype 1. In eight patients with phenotype 1,

seven had rearrangement of $V\beta 5.2$, and all eight MS patients rearranged with $V\beta 5.1$ or 5.2 , or both, compared with 2 of 7 MS brains from patients who were not phenotype 1 ($\chi^2 = 5.4$, $P < .025$). Other frequent $V\beta$ rearrangements seen in patients with phenotype 1 were $V\beta 6$, $V\beta 7$ and $V\beta 8$ in 6/8 patients, and $V\beta 12$ in 4/8 patients. In MBP-specific T cells from HLA DR2 MS patients, rearrangements of $V\alpha 1$, 2, 7, 8 and 10 were prominent¹². These same $V\alpha$ TCRs were rearranged in all seven phenotype 1 patients and were present in 13 of 17 plaques from these patients.

We next sequenced $V\beta 5.2$ rearrangements from two plaques from different anatomic regions in the brain of MS patient K.L., who was HLA phenotype 1. We also sequenced $V\beta 5.2$ rearrangements from another MS brain (patient L.J.) who was HLA phenotype 1. We found five predominant $V\beta 5.2$ motifs in brain K.L. (Table 1): (Q)LR or LR(5/35 sequences; one-letter amino-acid code), LGG(4/35), LVAG(4/35), LDG(3/35), and (Q)PT(3/35). The $V\beta 5.2$ LR, LVAG, and PT motifs were seen in both anatomic regions sampled from K.L. Moreover they were also seen in brain L.J. (Table 1). None of these sequences was seen in $V\beta 5.2$ sequences from a muscle biopsy of an individual with Duchenne muscular dystrophy who was phenotype 1, or from $V\beta 5.2$ sequences from peripheral blood lymphocytes of healthy individuals (J.O.: HLA DR3DRw8; B.M.: phenotype 1).

Rearrangements at the site of inflammation in the brains of three patients with encephalitis were studied. K.W. (HLA DRB1*0404,0901, DQB1*0302,0305, DPB1*0401,0402), a patient with viral encephalitis, rearranged $V\alpha 5$, 8, 10, 12, 14 and 16 and $V\beta 1$, 2, 3, 6, 11, 12, 13 and 15. S.E. (HLA DRB1*1201,1501, DQB1*0301,0602, DPB1*0401,0501), another patient with viral encephalitis, rearranged $V\alpha 2$, 3, 6, 8, 10, 12, 13, 14, 16, 17 and 18, and all $V\beta$ genes other than $V\beta 20$. S.P. (HLA DRB1*1303,11, DQB1*0301,0180, DPB1*0201,0401), with progressive multifocal leucoencephalopathy, rearranged $V\alpha 1$ to 10, 12, 13, 16, 17 and 18 and $V\beta 2$, 3, 9, 10 and 14 through to 20. TCR V region transcripts were in general more diverse in viral encephalitis than in MS. As patient S.E. was HLA phenotype 1, we sequenced $V\beta 5.2$ rearrangements from inflammatory lesions. There were no repeated sequences. We did not see the LR motif, though we did find one example of the LVAG sequence (Table 1).

One of the repeated motifs found in MS brains K.L. and L.J. contained the sequence $V\beta 5.2$ LCASSLRGA at the V-D-J junction, which is identical to that found in a T-cell clone recognizing MBP peptide 87–106 in an MS patient¹. We established that the HLA type of this patient is phenotype 1. This CDR3 sequence is also seen in T-cell clones isolated from the spinal cords of Lewis rats with experimental allergic encephalomyelitis(EAE). These clones are reactive to MBP peptide 87–99, and cause paralysis when adoptively transferred². The nucleotide sequences encoding the $V\beta 5.2$ LR motif in the K.L. and L.J. brain specimens reveal strong selection for these amino acids. It is unlikely that these sequences represent an artefact of complementary DNA cloning or PCR (Table 2). It is remarkable that the same CDR3 sequence, $V\beta 5.2$ LRGA, is selected in the brains of different MS patients, although different codons are used, and that these codons are in turn different from those encoding $V\beta 5.2$ LRGA in a T-cell clone recognizing MBP 87–106 (ref. 1).

The LR motif was described in other CDR3 sequences. A CDR3 sequence LRAG was found in a cDNA library derived from human tonsillar tissue¹³. A CDR3 sequence LCASSLLRS was seen in a human T-cell clone reactive to MBP peptide 139–153 plus HLA DR2a, whereas a CDR3 sequence, LCASS-REFSS, was observed in a human T-cell clone recognizing MBP peptide 80–99 and HLA DRB1*1501 (ref. 14). Another sequence, LRDF, is seen in a murine cytotoxic T-cell clone specific for influenza A (ref. 15). MBP and influenza share amino-acid sequences¹⁶. It may only be necessary for an

TABLE 1 CDR3 sequences of TCR rearrangements amplified from MS brains and controls

Vβ5.2/3	N-D-N-	J	Cβ		Vβ5.2/3	N-D-N-	J	Cβ	
KL-1					SE (viral encephalitis)				
LCASS	LPGTP	YGYFGSGTRLTVV	(β1.2)	EDLKN	LCASS	PCLFNR	GYGYTFGSGTRLTVV	(β1.2)	EDLKN
LCASS	LPGTP	YGYTFGSGTRLTVV	(β1.2)	EDLKN	LCASS	GRSGT	NYGTFGSGTRLTVV	(β1.2)	EDLKN
LCASS	LRLAN	SPLHFGNGTRLTVT	(β1.6)	EDLKN	LCASS	NQGH	YNSPLHFGNGTRLTVT	(β1.6)	EDLKN
LCASS	LDRL	YNSPLHFGNGTRLTVT	(β1.6)	EDLKN	LCASS	QATG	YNSPLHFGNGTRLTVT	(β1.6)	EDLKN
LCAS	QLRLA	NSPLHFGNGTRLTVT	(β1.6)	EDLKN	LCASS	RLDRGQGE	EQFFGPGTRLTVL	(β2.1)	EDLKN
LCASS	QLRLA	NSPLHFGNGTRLTVT	(β1.6)	EDLKN	LCASS	ITCGLAGARD	EQFFGPGTRLTVL	(β2.1)	EDLKN
LCASS	FLG	YNSPLHFGNGTRLVT	(β1.6)	EDLKN	LCASS	WGLAGARD	EQFFGPGTRLTVL	(β2.1)	EDLKN
LCASS	QPTV	YNNEQFFGQRTRLVL	(β2.1)	EDLKN	LCASS	TSGQP	GELFFEGGSRLTVL	(β2.2)	EDLKN
LCASS	SDGRM	STQYFGPTRLVL	(β2.3)	EDLKN	LCASS	LDLGA	DTQYFGPTRLTVL	(β2.3)	EDLKN
LCASS	LVAG	SIYEQYFGPTRLTVT	(β2.7)	EDLKN	LCASS	LVAG	KNIQYFGAGTRLSVLK	(β2.4)	EDLKN
LCASS	SEREG	RAQYFGQGTRLTVL	(β?)	EDLKN	LCASS	GTL	VLTFGAGSRLTVL	(β2.6)	EDLKN
LCASS	GGEQ	RAQYFGQGTRLTVL	(β?)	EDLKN	LCASS	YGTSGI	YEQYVGPTRLTVT	(β2.7)	EDLKN
					LCASS	FIWG	YEQYFGPTRLTVT	(β2.7)	EDLKN
					LCASS	TAGT	YEQYFGPTRLTVT	(β2.7)	EDLKN
KL-3					Muscle infiltrating lymphocytes				
LCASS	LDGVP	YGYTFGSGTGLTVV	(β1.2)	EDLKN	LCASS	LGSPGYR	TNEKLFFGSGTQLSVL	(β1.4)	EDLKN
LCASS	LDGVP	YGYTFGSGTRLTVV	(β1.2)	EDLKN	LCASS	FTGAY	YNEQFFGPGTRLTVL	(β2.1)	EDLKN
LCASS	LDGV	NYGYTFGSGTRLTVV	(β1.2)	EDLNIK	LCASS	RRTSGFVH	DTQYFGPTRLTVL	(β2.3)	EDLKN
LCASS	LVGRGP	YGYTFGSGTRLTVV	(β1.2)	EDLKN	LCAS	ARRTSGFV	TDTQYFGPTRLTVL	(β2.3)	EDLKN
LCASS	LGGVP	YGYTFGSGTGLTVV	(β1.2)	EDLKN	LCAS	TARRTSGFV	TDTQYFGPTRLTVL	(β2.3)	EDLKN
LCASS	LRGTP	YGYTFGSGTRLTVV	(β1.2)	EDLKN	LCA	TARRTSGFV	TDTQYFGPTRLTVL	(β2.3)	EDLKN
LCASS	QPAV	YNEQFFGPGTRLTVL	(β2.1)	EDLKN	LCA	TARRTSGFV	TDTQYFGPTRLTVL	(β2.3)	EDLKN
LCASS	LELAG	YNEQFFGPGTRLTVL	(β2.1)	EDLKN	LCA	TARRTSGFV	TDTQYFGPTRLTVL	(β2.3)	EDLKN
LCASS	LGGSEE	DTQYFGPTRLTVL	(β2.3)	EDLKN	LCA	TARRTSGFV	TDTQYFGPTRLTVL	(β2.3)	EDLKN
LCASS	LGGSE	ETQYFGPTRLVL	(β2.5)	EDLKN	LCA	TARRTSGFV	TDTQYFGPTRLTVL	(β2.3)	EDLKN
LCASS	LGGSV	ETQYFGPTRLVL	(β2.5)	EDLKN	LCA	TARRTSGFV	TDTQYFGPTRLTVL	(β2.3)	EDLKN
LCASS	LGSGL	QETQYFGPTRLVL	(β2.5)	EDLKN	LCAS	RQGART	GANVLTFGAGSRLTVL	(β2.6)	EDLKN
LCASS	LASGTL	QETQYFGPTRLVL	(β2.5)	EDLKN	JO (PBLs)				
LCASS	LASGTL	QETQYFGPTRLVL	(β2.5)	EDLKN	LCASS	VALQDR	YGYTFGSGTGLTVV	(β1.2)	EDLKN
LCASS	PT	GANVLTFGAGSRLTVL	(β2.6)	EDLKN	LCASS	TVRGS	QPQHFGDGTRLSIL	(β1.5)	EDLKN
LCASS	PT	GANVLTFGAGSRLTVL	(β2.6)	EDLKN	LCASS	PGM	KNIQYFGAGTRLSVL	(β2.4)	EDLKN
LCASS	QGS	TFGAGSRLTVL	(β2.6)	EDLKN	LCASS	DSPSG	QETQYFGPTRLTVL	(β2.5)	EDLKN
LCASS		SGANVLTFGAGSRLTVL	(β2.6)	EDLKN	LCASS	RPGNIR	ETQYFGPTRLTVL	(β2.5)	EDLKN
LCASS	L	GANVLTFGAGSRLTVL	(β2.6)	EDLKN	LCASS	RSQGART	GANVLTFGAGSRLTVL	(β2.6)	EDLKN
LCASS	LR	GANVLTFGAGSRLTVL	(β2.6)	EDLKN	BM (PBLs)				
LCASS	LVAG	SIYEQYFGPTRLTVT	(β2.7)	EDLKN	LCASS	DAG	YNSPLHFGNGTRLTVT	(β1.6)	EDLKN
LCASS	LVAG	SIYEQYFGPTRLTVT	(β2.7)	EDLKN	LCASS	YRTQL	NSPLHFGNGTRLTVT	(β1.6)	EDLKN
LCASS	LVAG	SIYEQYFGPTRLTVT	(β2.7)	EDLKN	LCASS	LEHRPT	AKNIQYFGAGTRLSVL	(β2.4)	EDLKN
					LCASS	PER	GANVLTFGAGSRLTVL	(β2.6)	EDLKN
					LCASS	QEA	SYEQYFGPTRLTVT	(β2.7)	EDLKN
					LCAS	RLVRDL	SHEQYFGPSTRLTVT	(β2.7)	EDLKN
LJ 1									
LCASS	TLRL	GNSPLHFGNGTRLTVT	(β1.6)	EDLKN					
LCASS	DSS	ETQYFGPTRLVL	(β2.5)	EDLKN					
LCASS	LR	GANVLTFGAGSRLTVL	(β2.6)	EDLKN					
LCASS	LR	GANVLTFGAGSRLTVL	(β2.6)	EDLKN					
LCASS	PT	GANVLTFGAGSRLTVL	(β2.6)	EDLKN					
LCASS	LVAG	IYEQYFGPTRLTVT	(β2.7)	EDLKN					
LCASS	LVAG	SIYEQYFGPSTRLTVT	(β2.7)	EDLKN					
LCASS	LVAG	SIYEQYFGPSTRLTVT	(β2.7)	EDLKN					

cDNA was amplified with $V\alpha$ - or $V\beta$ -specific primers as described³. The specificity of each primer pair has been confirmed^{3,4}. The $V\beta$ 5.2 primer does not discriminate between $V\beta$ 5.2 and $V\beta$ 5.3, but can distinguish them from $V\beta$ 5.1 and 5.4. ($V\beta$ 5.2/5.3 is referred to as $V\beta$ 5.2). Actin was amplified in all specimens as a positive control for cDNA integrity. The number of different TCR $V\alpha$ genes transcribed ranged from 0 to 9 per brain, with a mean of 4.4 ± 2.8 ($\pm$ s.d.). TCR $V\beta$ rearrangements were more diverse, showing a range of 2 to 13 different $V\beta$ families with rearranged transcripts per brain, mean 7.0 ± 3.4 ($\pm$ s.d.). TCR $V\alpha$ or $V\beta$ transcripts were detected in one of the 11 brains of individuals with non-neurological diseases or non-inflammatory neurological conditions; this patient had epilepsy. All MS samples and controls were coded, with investigators blind to specimen origin, except for 3 MS brains and 3 controls from which $V\alpha$ transcripts had been previously analysed⁴. All patients had chronic progressive MS, and no correlations with TCR V gene usage or HLA molecular phenotype could be made on the basis of sex, duration of disease, or the region of brain sampled. Sequencing of cDNA: Samples were amplified by PCR using $V\beta$ 5.2 primer³, cloned into M13, and digested with *Pst*I and *Sac*I, or with *Pst*I and *Bam*HI to cut restriction sites in the oligonucleotide primers. Centricon centrifugal microconcentrators (Amicon) were used to concentrate and desalt digested PCR products. After transformation in JM101-competent cells, clones containing TCR- β inserts were identified by hybridization with a TCR- β C-region horseradish peroxidase-labelled probe. Single-stranded DNA from positive clones was prepared and *VDJ β* sequences determined by dideoxy chain termination using an AmpliTaq sequencing kit (Perkin-Elmer).

infectious pathogen to share a few amino acids within an epitope to provoke a pathogenic crossreaction. EAE could be induced with only four core amino acids out of an 11-amino-acid peptide of MBP¹⁷.

Another motif found in plaque KL-3 was LCASSLGGSEET (Tables 1 and 2). In the Lewis rat some T cell clones, isolated from lymph nodes, and specific for MBP peptide 87-99 have a CDR3 sequence of LCASSLGGSEET². In plaque KL-1, a CDR3 sequence LCASSGEG was seen (Table 1). Another motif LVAG was also evident in the CDR3 region (IAG) of T-cell clones which are specific for MBP peptide 87-99 and cause EAE

(Tables 1 and 2). Taken together, the presence and frequency (16/40 unique sequences, not counting any repeated sequences which might represent sister clones) of these three CDR3 motifs, LCAS(S)LRG, LCASSLVAG and LCASSLGG(S)E, suggest that an immune response to an epitope within MBP peptide 87-106 may be occurring in MS lesions. This immune response may be deleterious, given that T cells recognizing this epitope of MBP cause paralysis in the EAE model².

Although both α - and β -chains of TCR contribute to and define the peptide-MHC specificity of a T cell¹⁸, the identification of TCR- β CDR3 sequences identical to those found

TABLE 2 Nucleotide sequence homology in the use of Leu-Arg-Gly, Leu-Gly-Gly-Glu and Leu-Val-Ala-Gly

Sample	N-D-N-J			
KL3	AGCAGC	CTACGCGGGGCCAAC	SS LRGAN	(Vβ5.2/Jβ2.6)
	AGCAGC	TTACGCGGGACACCC	SS LRGT	(Vβ5.2/Jβ1.2)
KL1	AGCAGC	TTGGCGCTTGGCTAAT	SS LRLAN	(Vβ5.2/Jβ1.6)
	AGC	CAGTTGGCGCTTGGCTAAT	S Q LRLA	(Vβ5.2/Jβ1.6)
	AGCAGC	CAGTTGGCGCTTGGCTAAT	SS Q LRLA	(Vβ5.2/Jβ1.6)
	AGCAGC	TTGGATCGCTTGTATAAT	SS LDRLA	(Vβ5.2/Jβ1.6)
LJ1	AGC	ACGTTGGCGCTTGGGT	S T LRLG	(Vβ5.2/Jβ1.6)
	AGCAGC	CTACGCGGGGCCAAC	SS LRGAN	(Vβ5.2/Jβ2.6)
	AGCAGC	CTACGCGGGGCCAAC	SS LRGAN	(Vβ5.2/Jβ2.6)
MS18*	AGCAGC	TTGAGGGGGGCGCTA	SS LRGA	(Vβ5.2/Jβ2.4)
BF1†	AGCAGC	CTCAGGGGG	SS LR	(Vβ6/Jβ1.6)
E†	AGCAGC	ATAAGGGGAAGC	SS IRGS	(Vβ6/Jβ2.7)
BD3‡	AGCAGC	ATCGTCAGGGGATCG	SS IVRGS	(Vβ6/Jβ2.7)
ph 11‡	AGCAGT	TTAAGGGCGGGA	SS LRAG	(Vβ8/Jβ1.1)
12H6§	AGCAGC	CTCGGGGACTTT	SS LRDF	(Vβ13/Jβ2.1)
KL3	AGCAGC	TTGGGAGGGTACCTAT	SS LGGVPY	(Vβ5.2/Jβ1.2)
	AGCAGC	TTGGGAGGGTCCGAAGAG	SS LGGSEE	(Vβ5.2/Jβ2.3)
	AGCAGC	TTGGGAGGGTCCGAAGAG	SS LGGSEE	(Vβ5.2/Jβ2.5)
	AGCAGC	TTGGGAGGGTCCGTTGAG	SS LGGSE	(Vβ5.2/Jβ2.5)
4	AGCAGC	CTGGGGGGCGAA	SS LGGE	(Vβ8.2/Jβ2.5)
KL3	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(Vβ5.2/Jβ2.7)
	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(Vβ5.2/Jβ2.7)
	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(Vβ5.2/Jβ2.7)
KL1	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(Vβ5.2/Jβ2.7)
LJ1¶	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(Vβ5.2/Jβ2.7)
	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(Vβ5.2/Jβ2.7)
C†	AGCAGC	ATAGCTGGCGGT	SS IAGG	(Vβ6/Jβ2.3)

* CDR3 usage in human MBP 88-99 specific T-cell line¹.

† CDR3 usage in rat spinal cord derived T-cell clones specific for MPB 85-99 (ref. 2).

‡ Clone derived from a human tonsil cDNA library¹³.

§ Non-cytolytic mouse T-cell clone specific for influenza virus strain A/PR8/34 (ref. 15).

|| CDR3 usage in rat lymph node derived T-cell clone specific for MBP 85-99 (ref. 2).

¶ G→S substitution in Jβ2.7.

MHC class II-positive epithelium and mesenchyme cells are both required for T-cell development in the thymus

Graham Anderson, Eric J. Jenkinson, Nel C. Moore & John J. T. Owen

Department of Anatomy, Medical School, University of Birmingham, Birmingham B15 2TT, UK

T LYMPHOCYTES are produced in the thymus from precursors originating in the haemopoietic tissues. On entering the thymus, they undergo a programme of proliferation, T-cell receptor (TCR) gene rearrangement, differentiation and repertoire selection¹. Although the thymus provides a unique environment for these events, the role of the thymic stroma in regulating specific developmental stages is not well understood². We therefore devised an *in vitro* system to study the role of individual thymic stromal components in T-cell development. We report here that the development of TCR⁺CD4⁺CD8⁺ T-cell precursors into TCR⁺ cells expressing CD4 and/or CD8 requires the presence of both major histocompatibility complex class II⁺ epithelial cells and fetal mesenchyme. The requirement for mesenchymal support can be mapped to the initial stages of intrathymic development because the later stages of maturation, from double-positive CD4⁺CD8⁺ thymocytes into single-positive CD4⁺ or CD8⁺ cells, can be supported by epithelial cells alone. We also show that the requirement for mesenchymal cells can be met by cells of the fibroblast line 3T3 (but not by supernatants from these cells). To our knowledge, these findings provide the first direct evidence that mesenchymal as well as

in bona fide clones with specificity for MBP 87-106 suggests strongly that T cells of such antigenic specificity are present in MS brain lesions, and that they represent a substantial fraction of the cells expressing Vβ5.2 TCR. An immune response directed against other antigens such as proteolipid protein, myelin-oligodendroglial glycoprotein and heat-shock proteins may also be critical in MS. As sequences for TCRs specific for these antigens become available, attribution of the specificity of some of the other rearrangements seen within lesions may be possible. □

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epithelial cells are involved in T-cell development, and suggest that their involvement is stage-specific and likely to be dependent on short-range or contact-mediated interactions.

TABLE 1 Thymic stromal cell requirements for T-cell development *in vitro*

Stromal cells	Thymocyte input	T-cell development in reaggregate cultures
Whole dGuO-treated stroma	14d TCR ⁺ CD4 ⁺ CD8 ⁺	2/2*
Whole dGuO-treated stroma depleted of class II ⁺ epithelium	14d TCR ⁺ CD4 ⁺ CD8 ⁺	0/3†
Purified class II ⁺ epithelium	14d TCR ⁺ CD4 ⁺ CD8 ⁺	0/5†
Purified class II ⁺ epithelium and fetal mesenchyme	14d TCR ⁺ CD4 ⁺ CD8 ⁺	3/3*
Purified class II ⁺ epithelium and 3T3 Fibroblasts	14d TCR ⁺ CD4 ⁺ CD8 ⁺	2/2*
Purified class II ⁺ epithelium	TCR ⁺ CD4 ⁺ CD8 ⁺	3/3‡
Whole dGuO-treated stroma depleted of class II ⁺ epithelium	TCR ⁺ CD4 ⁺ CD8 ⁺	0/2†

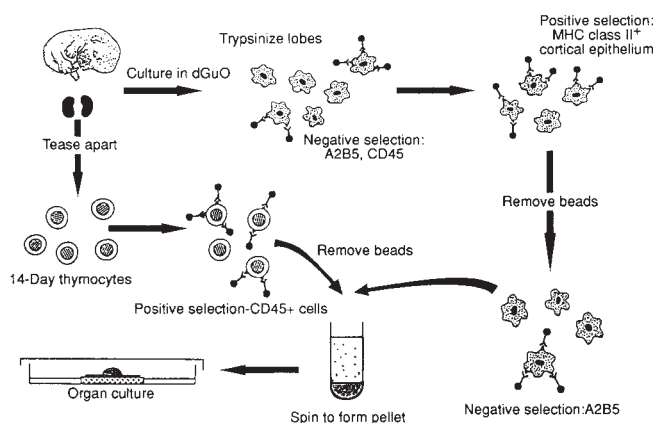
After 9 days, individual reaggregate cultures were gently teased apart to liberate any lymphoid cells. T-cell development was assessed on lymphoid content, and by CD4, CD8 and TCR expression, determined by three-colour flow cytometry (see legend to Fig. 3 for details). dGuO, deoxyguanosine.

* Cell yields in successful reaggregate cultures showed a 2-5-fold increase over the lymphoid cell number added at the start of the culture. This probably represents an underestimate of the proliferative capacity of 14-day precursors because it is likely that not all cells are successfully incorporated into the reaggregates.

† Cultures recorded as unsuccessful contained less than 10% of the original input number.

‡ Cell yields from 4-day cultures of CD4⁺CD8⁺TCR⁺ cells and purified epithelium were 20-30% of the input number, reflecting the limited life span of these cells unless rescued by positive selection¹⁴.

FIG. 1 Techniques for preparing reaggregate organ cultures from selected thymocyte and thymic stromal cell populations. Thymic stromal cells were prepared by disaggregating deoxyguanosine (dGuO)-treated BALB/c fetal thymus lobes using 0.25% trypsin, 0.02% EDTA in Ca^{2+} and Mg^{2+} -free PBS. Residual cells of haemopoietic origin were removed from the suspension by three rounds of depletion using anti-CD45 (clone M1-9, ATCC)-coated anti-rat immunoglobulin magnetic beads (Dynal). The ability of anti-CD45-coated beads to bind to CD45^{+} cells is not prevented by prior trypsinization (our unpublished observations). To promote interaction, cells and beads were spun together in round-bottomed freezing vials (Sterilin) as described previously⁷. Stromal preparations lacking MHC class II⁺ epithelial cells were obtained by immunomagnetically depleting suspensions using three rounds of anti-IaD (clone MK-D6, Becton-Dickinson) coated onto anti-mouse immunoglobulin beads. To prepare purified class II⁺ epithelial cells, suspensions of CD45-depleted dGuO stroma were first immunomagnetically depleted of cells expressing the medullary marker A2B5 using anti-rat immunoglobulin beads coated sequentially with rat anti-mouse IgM (Pharmingen) and A2B5 hybridoma supernatant (a gift from M. Raff). Successful rosetting of A2B5⁺ cells with antibody-coated beads was monitored by microscopy, confirming that A2B5 (a GQ ganglioside¹⁵) is protease-resistant. To obtain maximum depletion, beads and cells were spun together at a ratio of 5:1 in two successive rounds. Within each round, cells and beads were spun and resuspended twice. Rosetted cells and free beads were removed on a magnet after each round. MHC class II⁺ epithelial cells were then positively selected from the remaining suspension using anti-IaD-coated beads at a bead:cell ratio of 2:1. Rosetted cells were collected on a magnet and washed four times to remove any unbound cells. After a final wash in PBS, rosettes were resuspended in 200 μl pronase (10 mg ml^{-1} in PBS; Sigma) at 37 °C for 2 min to remove the beads from the cells. The reaction was stopped by adding 800 μl ice-cold medium containing 10% fetal calf serum (FCS) and the released beads plus any residual rosettes removed on a magnet. Free cells were collected by centrifugation and subjected to a further depletion with A2B5-coated beads as above. Reanalysis by flow cytometry showed these preparations to be free of A2B5⁺ cells (data not shown). Fetal mesenchyme cells were prepared from the capsule of the fetal lung rudiment to avoid the possibility of contamination with non-mesenchymal thymic stroma. Isolated 14-day rudiments were incubated in 0.25% trypsin, 0.02% EDTA for 5 min, or until the outer mesenchymal



capsule was just beginning to dissociate. Gentle pipetting then released these cells leaving the remainder of the rudiment largely intact. 3T3 cells were grown as monolayers in flasks, collected by trypsinization and allowed to stand at 4 °C for 2 h before incorporation into reaggregates. $\text{TCR}^{-}\text{CD4}^{-}\text{CD8}^{-}$ T-cell precursors were obtained by gently teasing apart freshly isolated 14-day fetal thymus lobes. The resultant suspensions were then positively selected using anti-CD45-coated beads as described above, except that beads were removed from rosetted cells using Detachabead (Dynal) according to manufacturer's instructions. This selection removes the possibility of contamination by fixed thymic stromal elements, although some cells or precursors of other haemopoietic lineage may be included in the selected population. Reaggregates were formed by mixing together the desired stromal and lymphoid components (usually at a ratio of 1:2 for epithelium and lymphocytes, and 2:4:1 for epithelium, lymphocytes and fibroblasts) and pelleting by centrifugation. After removal of the supernatant, the pellet was dispersed into a slurry, drawn into a fine glass pipette and placed as a standing drop on the surface of a nucleopore filter in organ culture. Within 12 h, intact thymic lobes reform from these mixtures (see Fig. 2).

Previous *in vitro* attempts to investigate the role of thymic stromal cells in T-cell development have been hampered by the limited ability of monolayer cultures to support a full programme of T-cell maturation and by the difficulty of obtaining cultures of thymic epithelial cells that maintain their *in vivo* phenotype^{3,4}. We devised a technique (Fig. 1) to reaggregate defined thymocyte and stromal cell populations under organ culture conditions that provide optimal support for T-cell development *in vitro*⁵. Stromal cells were prepared by immunomagnetic selection from disaggregated 14-day fetal thymus lobes rendered free of lymphoid and major histocompatibility complex (MHC) class II⁺ dendritic cells by culture in deoxyguanosine⁶. These lobes, after depletion of remaining CD45^{+} haemopoietic elements, provide a source of MHC class II⁺ cortical epithelial cells, medullary epithelial cells and mesenchymal components⁷. Day-14 fetal thymocytes (CD45^{+} , $\alpha\beta$ TCR^{-} , >97% $\text{CD4}^{-}\text{CD8}^{-}$) were used as a source of T-cell precursors, and purified $\text{CD4}^{+}\text{CD8}^{+}\text{TCR}^{-}$ cells provided an intermediate stage of thymocyte development. Mixtures of thymocytes and stromal cells rapidly reform (within 12 hours) into intact thymus lobes and were analysed for T-cell development in terms of lymphoid content and TCR, CD4 and CD8 expression after 9 days in culture (Fig. 2).

The results of associating various combinations of thymocytes and stromal cells are summarized in Table 1. In contrast to reaggregates using whole stromal cell preparations, lymphocytes were not recovered from reaggregate cultures of 14-day precursors and stromal preparations previously depleted of class II⁺ epithelial cells. Purified class II⁺ epithelial cells also failed to support precursor development, even though the epithelial cells reaggregated successfully (Fig. 2a, left) and retained a cortical epithelial phenotype including MHC class II expression (Fig. 2b). Such lobes remained small and were alymphoid on

collection. But when fetal mesenchyme was also incorporated, the reaggregates increased in size (Fig. 2a, right) and lymphoid content, and supported the development of $\alpha\beta$ TCR^{+} T cells expressing CD4 and CD8 (Fig. 3a, b).

Taken together, these results provide direct evidence that MHC class II⁺ epithelial cells are necessary but not sufficient for T-cell development and show that, in addition to any non-lymphoid cells in the CD45^{+} input, mesenchymal elements are also required. But when double-positive $\text{CD4}^{+}\text{CD8}^{+}\text{TCR}^{-}$, rather than $\text{CD4}^{-}\text{CD8}^{-}\text{TCR}^{-}$ cells were reaggregated with purified epithelium, further development did occur, resulting in the appearance of double-positive TCR^{+} and single-positive CD4^{+} and CD8^{+} cells with upregulated levels of TCR expression (Fig. 3e-h). This pattern of differentiation *in vitro* parallels that seen as a result of TCR-mediated positive selection *in vivo*, and is accompanied by the development of functionally competent cells⁷. Thus the requirement for mesenchyme seems to be associated with the early stages of thymopoiesis, whereas epithelial cells alone can support the later developmental stages, including those dependent on TCR-mediated interactions with MHC antigens in the thymic stroma. In addition, the failure of class II-depleted thymic stromal cells to support the development of $\text{CD4}^{+}\text{CD8}^{+}\text{TCR}^{-}$ cells (Table 1) indicates that class II⁺ epithelial cells are essential, as well as being sufficient for these later stages of thymocyte maturation.

Because fetal mesenchyme is likely to consist of a heterogeneous mixture of cells, we investigated the mesenchymal components required for the initial stages of thymopoiesis. Therefore we examined the ability of 3T3 cells (a murine fibroblast cell line) to substitute for fetal mesenchyme. A combination of purified class II⁺ epithelial cells and 3T3 cells (Fig. 3c, d) was able to support the maturation of 14-day precursors, but supernatants from confluent 3T3 cultures were not. These

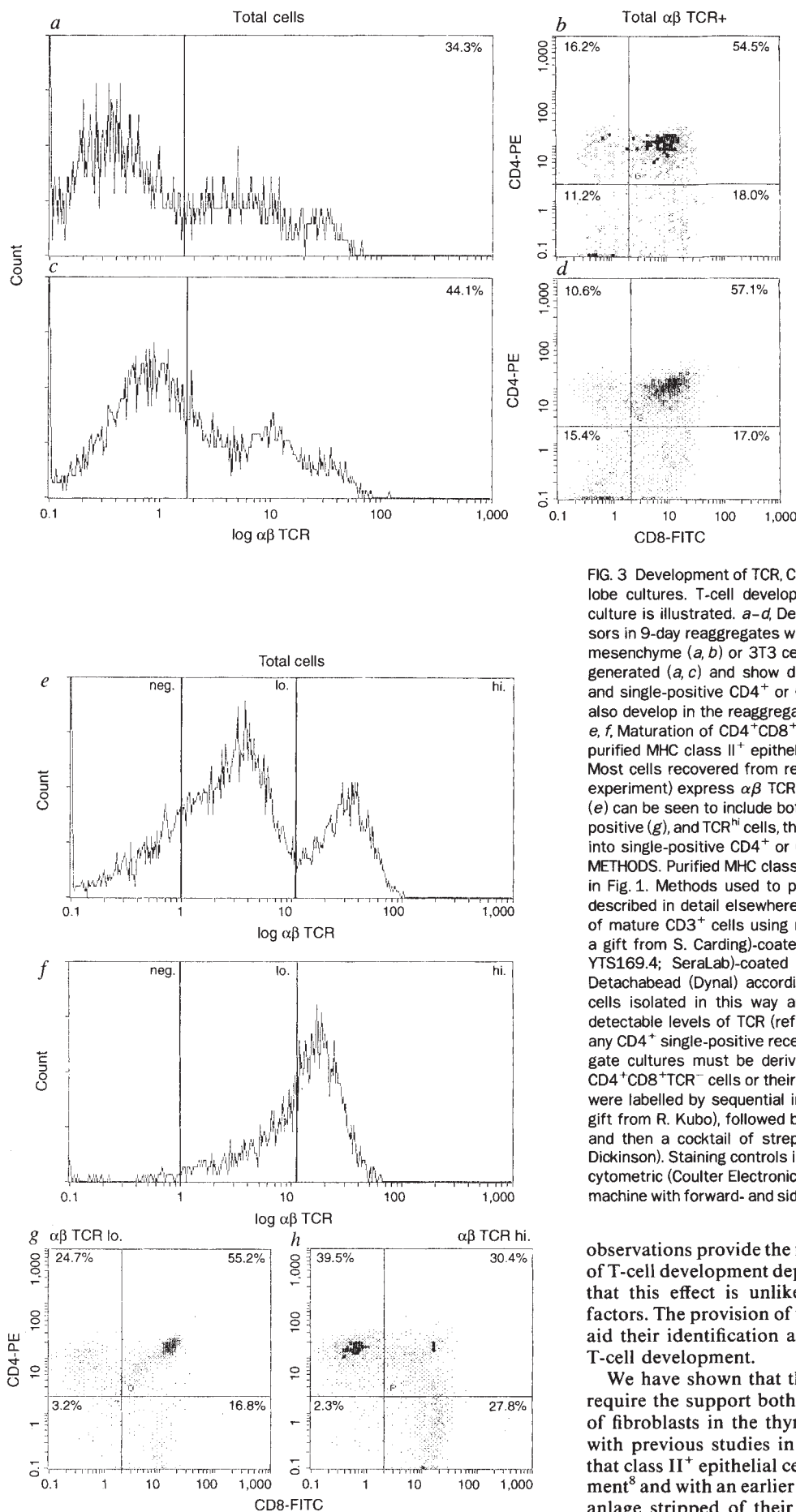


FIG. 3 Development of TCR, CD4 and CD8 expression in reaggregate thymus lobe cultures. T-cell development in two different types of reaggregate culture is illustrated. *a-d*, Development of $CD4^+CD8^-TCR^-$ 14-day precursors in 9-day reaggregates with purified class II⁺ epithelium and either fetal mesenchyme (*a, b*) or 3T3 cells (*c, d*). In both cases, TCR^+ cells have been generated (*a, c*) and show differentiation into double-positive $CD4^+CD8^+$ and single-positive $CD4^+$ or $CD8^+$ cells (*b, d*). (Note, $\gamma\delta$ TCR^+ thymocytes also develop in the reaggregate cultures described above; data not shown.) *e, f*, Maturation of $CD4^+CD8^+TCR^-$ cells in 4-day reaggregate cultures with purified MHC class II⁺ epithelium as the only type of stromal cell present. Most cells recovered from reaggregates (25% of the input number in this experiment) express $\alpha\beta$ TCR (*f*) and by comparison with adult thymocytes (*e*) can be seen to include both TCR^{lo} cells which are predominantly double-positive (*g*), and TCR^{hi} cells, the majority of which have undergone maturation into single-positive $CD4^+$ or $CD8^+$ cells (*h*).

METHODS. Purified MHC class II⁺ epithelial cells were isolated as described in Fig. 1. Methods used to purify $CD4^+CD8^+TCR^-$ thymocytes have been described in detail elsewhere⁷. Briefly, newborn thymocytes were depleted of mature $CD3^+$ cells using multiple rounds of anti-CD3 (clone C363.29B; a gift from S. Carding)-coated beads, then selected using anti-CD8 (clone YTS169.4; SeraLab)-coated beads. Bead removal was achieved using Detachabead (Dynal) according to the manufacturer's instructions. TCR^- cells isolated in this way are >99.7% $CD4^+CD8^+$ and do not express detectable levels of TCR (ref. 7, and data not shown). Using this approach, any $CD4^+$ single-positive receptor-bearing cells generated in these reaggregate cultures must be derived from precursors expressing CD8, that is, $CD4^+CD8^+TCR^-$ cells or their $CD4^+CD8^+TCR^-$ precursors. Cell suspensions were labelled by sequential incubations in anti- $\alpha\beta$ TCR (clone H.57-597; a gift from R. Kubo), followed by anti-hamster immunoglobulin biotin (Caltag) and then a cocktail of streptavidin APC, CD4-PE and CD8-FITC (Becton-Dickinson). Staining controls involved omission of first-step antibodies. Flow cytometric (Coulter Electronics, FL) analysis was done on an Elite Dual laser machine with forward- and side-scatter gates set to exclude non-viable cells.

observations provide the first direct evidence that the early stages of T-cell development depend on fibroblast products and suggest that this effect is unlikely to be mediated solely by soluble factors. The provision of these requirements by a cell line should aid their identification and analysis of their functional role in T-cell development.

We have shown that the initial stages of T-cell development require the support both of MHC class II⁺ epithelial cells and of fibroblasts in the thymic stroma. Our results are consistent with previous studies in T-cell-deficient nude mice suggesting that class II⁺ epithelial cells play a crucial role in T-cell development⁸ and with an earlier report showing that fetal day-12 thymic anlage stripped of their mesenchymal capsule fail to support

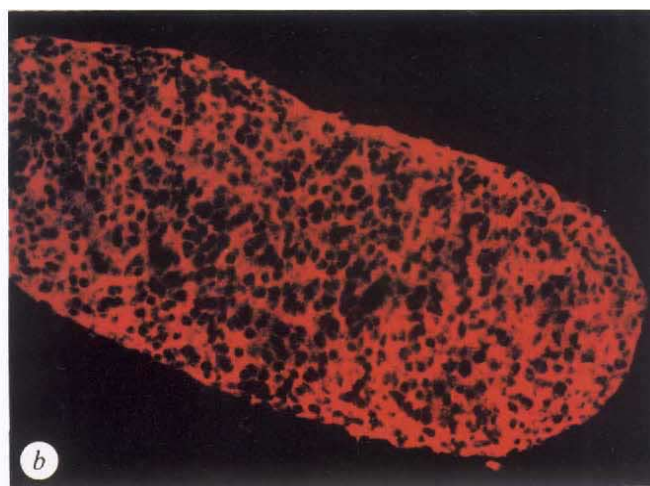
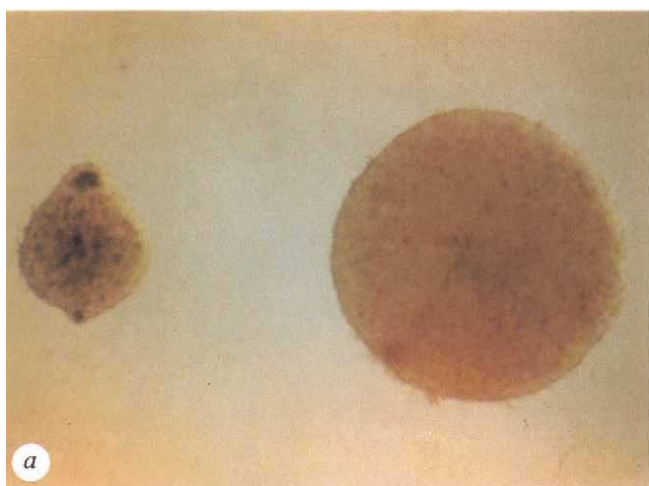


FIG. 2 Reaggregate cultures only support the development of 14-day CD4⁺CD8⁺TCR⁺ thymic precursors when both epithelial and mesenchymal cells are present. **a**, Two age-matched reaggregate cultures, prepared as in Fig. 1. The lobe on the right was prepared by reaggregating 14-day thymocytes, purified class II⁺ epithelial cells and fetal mesenchyme cells, whereas the other was prepared using 14-day thymocytes and purified epithelial cells alone. During the 9-day culture period, the lobe receiving mesenchyme cells increased in size and when collected showed a fourfold increase over the number of precursors added at the initiation of culture. Cells collected from such lobes show evidence of T-cell maturation comparable to that seen in unmanipulated cultures (Fig. 3). In contrast, the culture of precursors and purified epithelium alone remained small and was atypical, even though the epithelial cells had successfully formed an intact

structure and still expressed a cortical epithelial cell phenotype, including MHC class II antigen expression (**b**) and ERTR4 (ref. 16) and 4F11E (ref. 17) expression (not shown). In addition, such lobes were found to be negative for the macrophage markers 5C6 and F4/80 (ref. 18) and the reticular fibroblast marker ERTR7 (ref. 16).

METHODS. Reaggregate cultures were prepared as described for Fig. 1. For immunohistological analysis, cultures were embedded in OCT compound (Raymond Lamb, London) and snap-frozen in liquid nitrogen. Frozen sections were incubated sequentially in anti-mouse I-Ad (Becton-Dickinson) and alkaline phosphatase-conjugated anti-mouse immunoglobulin (Dako) with intervening washes. Slides were developed using a Fast Red TR substrate (Sigma) and viewed under fluorescence conditions as described previously¹⁹.

lymphocyte development⁹. They may also partly explain the limited success of attempts to induce a full programme of T-cell development using either monolayer cultures of a single thymic stromal cell type¹⁰, or cocktails of cytokines^{11,12}. Having identified two key cell types required for T-cell development, our studies also provide a basis for an analysis of the cellular products regulating specific developmental stages. Epithelial cells and fibroblasts differ in their pattern of cytokine gene expression²⁰, but it seems likely that fibroblasts may also influence T-cell maturation by contact mediated mechanisms. This might involve a direct effect on T-cell precursors, perhaps reflecting a continuation of the dependence of stem cells in the bone marrow on contact with fibroblasts or their matrix products¹³. Alternatively, indirect effects involving the regulation of epithelial cell function by mesenchyme are also possible. □

Decoration of the microtubule surface by one kinesin head per tubulin heterodimer

Beth C. Harrison*, Silvio P. Marchese-Ragona*†, Susan P. Gilbert*, Naiqian Cheng‡, Alasdair C. Steven‡ & Kenneth A. Johnson*§

* Department of Molecular and Cell Biology, Pennsylvania State University, University Park, Pennsylvania 16802, USA

† Laboratory of Structural Biology, National Institute of Arthritis, Musculoskeletal and Skin Diseases, NIH, Bethesda, Maryland 20892, USA

KINESIN, a microtubule-dependent ATPase, is believed to be involved in anterograde axonal transport. The kinesin head, which contains both microtubule and ATP binding sites, has the necessary components for the generation of force and motility¹. We have used saturation binding and electron microscopy to examine the interaction of the kinesin motor domain with the microtubule surface and found that binding saturated at one kinesin head per tubulin heterodimer. Both negative staining and cryo-electron microscopy revealed a regular pattern of kinesin bound to the microtubule surface, with an axial repeat of 8 nm. Optical diffraction analysis of decorated microtubules showed a strong layer-line at this spacing, confirming that one kinesin head binds per tubulin heterodimer. The addition of Mg-ATP to the microtubule-kinesin complex resulted in the complete dissociation of kinesin from the microtubule surface.

The kinesin ATPase has a low rate of ATP hydrolysis which is stimulated ~1,000-fold by microtubules²⁻⁵. The precise

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† Present address: TopoMetrix Application Laboratory, 1505 Wyatt Drive, Santa Clara, California 95054, USA.

§ To whom correspondence should be addressed.

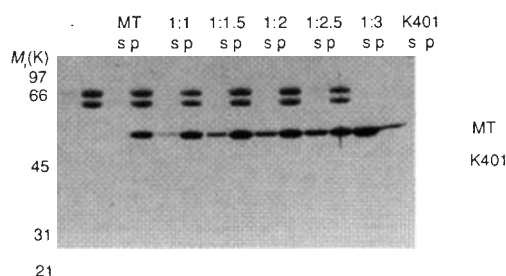


FIG. 1 Saturation binding of K401 to the microtubule surface. Microtubule-K401 complexes were formed in the absence of ATP or nucleotide analogues at molar concentration ratios of microtubule:K401 of 1:1, 1:1.5, 1:2, 1:2.5 and 1:3, with a microtubule concentration (tubulin heterodimer) fixed at

structural basis for interaction of these proteins, however, has yet to be defined. In these studies, the interaction of the kinesin head with the microtubule surface lattice was investigated by electron microscopy using a truncated form of the *Drosophila* kinesin heavy chain¹³. The truncated kinesin, K401, terminates at amino acid 401 and contains both the microtubule- and ATP-binding domains. K401 was expressed as soluble protein and purified to homogeneity in milligram amounts. The purified protein was fully active and demonstrated the steady-state kinetic properties expected of the native kinesin ATPase¹³: namely, a low ATPase in the absence of microtubules and a 1,000-fold stimulation upon the addition of microtubules. Because K401 consists of a single motor domain which is unable to dimerize, potential complications due to multiple microtubule binding sites were eliminated.

To determine the appropriate concentration of kinesin necessary to saturate the microtubule surface lattice, complexes were formed by mixing taxol-stabilized microtubules (1.5 μ M expressed as concentration of tubulin heterodimers) and K401 at 1.5–4.5 μ M (1 to 3 times the molar concentration of microtubule heterodimers) in the absence of ATP or nucleotide analogues. The microtubules were then sedimented with any bound K401 while free or excess kinesin remained in the supernatant. Samples were resolved by polyacrylamide gel electrophoresis (Fig. 1). At a ratio of one K401 per tubulin heterodimer, no excess K401 was apparent in the supernatant. As the concentration of K401 relative to tubulin was increased from 1:1 to 3:1, there was an increase in the amount of K401 in the supernatant and of K401 cosedimenting with the microtubules. Although it appeared that a fraction of the K401 was pelleting nonspecifically as the protein concentration was increased, the data suggest that saturation of the microtubule surface occurred at a ratio of one K401 molecule bound per tubulin heterodimer.

The interaction of K401 with the microtubule surface was visualized by electron microscopy to clarify the saturating stoichiometry. By conducting these studies with K401, we could observe the binding of single motor domains to the microtubule surface. The complex was formed by mixing taxol-stabilized microtubules with a slight molar excess of K401 in the absence of nucleotides, and the sample was examined by electron microscopy. Negatively stained specimens showed that the microtubules were heavily decorated on their outer surface (Fig. 2a), and had an axial periodicity of ~ 8 nm. A similar pattern was visible on frozen, hydrated specimens (Fig. 3b). The repeat is most evident along the edges of the microtubules, which are fringed by bound K401 molecules. Because the axial repeat per tubulin heterodimer in the microtubule surface lattice is 8 nm^{6,7}, these results support the interpretation of one kinesin motor domain per tubulin heterodimer.

Optical diffraction patterns from micrographs of kinesin-decorated microtubules show a strong helical layer line at an axial spacing of (8 nm)⁻¹ (Fig. 3d). This layer line is not visible

1.5 μ M. Samples were centrifuged and the resulting supernatants (s) and pellets (p) are shown for each microtubule-K401 ratio as well as for samples containing microtubules and 4.5 μ M K401 protein alone. Molecular weight standards as well as the locations of α - and β -tubulin (MT) and K401 are labelled.

METHODS. K401 and tubulin were prepared as described¹³. The plasmid containing the truncated kinesin gene (pET5b-K401) was transformed into *Escherichia coli* strain HMS174(DE3). After forming the microtubule-K401 complexes at room temperature for 5 min in HEPES ATPase buffer (20 mM HEPES, pH 7.2, 5 mM Mg-acetate, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 50 mM potassium acetate), samples were transferred to centrifuge tubes (cellulose propionate; Beckman) coated with Sigmacote (Sigma) to reduce the nonspecific sedimenting of K401 in the tube. Samples were then centrifuged at $\sim 120,000g$ in a Beckman Airfuge for 2 min. Supernatants and pellets were suspended in sample buffer and electrophoresed on an 8% acrylamide/2M urea gel in buffers described in ref. 10. The gel was stained with Coomassie brilliant blue R-250.

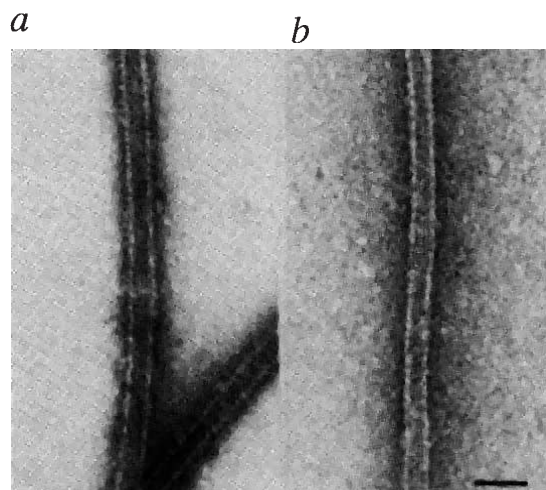


FIG. 2 Electron micrographs of the microtubule-K401 complex in the absence and presence of ATP. a, K401 decoration of microtubules in the absence of ATP. b, Dissociation of K401 from the microtubule surface upon the addition of ATP. Scale bar, 50 nm.

METHODS. The microtubule-K401 complex was formed at room temperature for 5 min with 0.67 μ M microtubules (tubulin heterodimer) and 0.86 μ M K401. HEPES ATPase buffer was added to one sample of the microtubule-K401 complex while 100 μ M Mg-ATP was added to a second sample. After incubation for 5 min at room temperature, protein solutions were placed on carbon- and formvar-coated grids. Grids were then negatively stained with 2% uranyl acetate and examined by transmission electron microscopy (JEOL 1200 EXII).

in patterns from undecorated control microtubules (Fig. 3c), whose most prominent layer line is at (4 nm)⁻¹ because the α - and β -tubulin monomers are indistinguishable at low resolution^{6,7}. In principle, the observed binding stoichiometry could represent either a coherent binding pattern of one kinesin per tubulin heterodimer, or alternatively, random binding of kinesin to α - and β -tubulin monomers, with saturation occurring at $\sim 50\%$ occupancy as a result of steric hindrance. The optical diffraction data, however, support the former alternative.

When Mg-ATP was added to the microtubule-kinesin complex, K401 decoration of the microtubule surface was eliminated (Fig. 2b). These results show conclusively that ATP promotes dissociation of kinesin from the microtubule surface. These observations are not necessarily in conflict with those showing the binding of kinesin to microtubules in the presence of non-hydrolysable ATP analogues^{2,4}. Hydrolysis of ATP may be required for dissociation or alternatively, the analogues may fail to induce the conformational change required for the release of

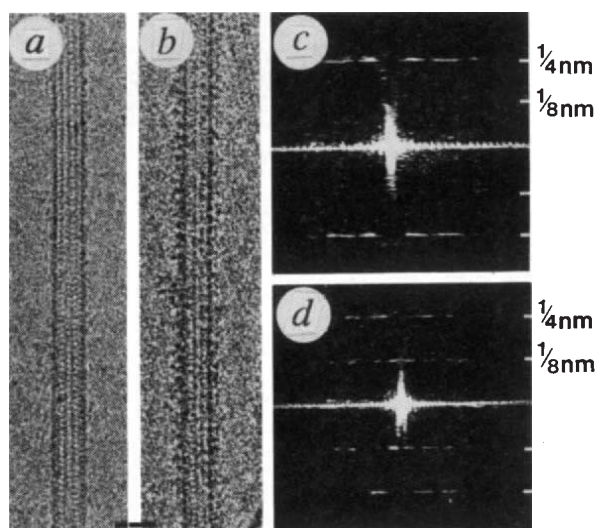


FIG. 3 Cryo-electron micrographs and optical diffraction patterns of undecorated and K401-decorated microtubules. Micrographs of *a*, microtubule in the absence of K401, and *b*, microtubule-K401 complex are shown with the corresponding optical diffraction patterns (*c* and *d*, respectively). Scale bar, 30 nm.

METHODS. The microtubule-K401 complex was prepared as described for Fig. 2 and was vitrified for observation by cryo-electron microscopy by quenching in liquid ethane. The procedures followed and the instrumentation used for specimen preparation, cryo-transfer and low-dose imaging were essentially as described¹¹; optical diffraction is described in ref. 12.

kinesin from the microtubule surface. The failure of kinesin to bind to microtubules in the presence of Mg-ATP has also been observed for a 45K fragment of the kinesin heavy chain^{8,9}. The microtubule-stimulated ATPase activity of this fragment, however, was much higher than that of intact kinesin or K401. It is possible that proteolysis of the kinesin heavy chain resulted in a change in the protein, leading to the elevated ATPase activity. In contrast, the K401 protein used in these studies exhibits ATPase activity comparable to that of native kinesin.

These results provide information concerning the interaction of the kinesin motor domain with microtubules. In the absence of Mg-ATP, saturation binding of K401 occurs at one kinesin head per tubulin heterodimer with a resulting periodicity of 8 nm. The addition of Mg-ATP to the microtubule-K401 complex produced the dissociation of K401 from the microtubule surface, suggesting that ATP promotes the release of the kinesin head from the microtubule during the force production cycle. Moreover, the data demonstrate that the head fragment truncated to 401 amino acids contains the structural elements necessary for interaction with ATP and the microtubule. □

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Alzheimer amyloid protein precursor complexes with brain GTP-binding protein G_o

Ikuko Nishimoto^{*†}, Takashi Okamoto^{*†},
Yoshiharu Matsuura[‡], Shuji Takahashi[§],
Toshimi Okamoto^{*†}, Yoshitake Murayama^{*}
& Etsuro Ogata^{*}

^{*} Life Science Laboratory, Fourth Department of Internal Medicine, University of Tokyo School of Medicine, 3-28-6 Mejirodai, Bunkyo-ku, Tokyo 112, Japan

[‡] Laboratory of Hepatitis Viruses, Department of Virology II, National Institute of Health, 1-23-1 Toyama, Shinjuku-ku, Tokyo 160, Japan

[§] Department of Pathology, Sapporo Medical College, S1, W17, Chuo-ku, Sapporo 060, Japan

THE most characteristic change in progressive dementia of Alzheimer's type is a tissue deposit of amyloid β/A_4 protein¹, which is derived from its precursor protein APP (ref. 2). Structural alterations of APP are implicated in the pathogenesis of Alzheimer's disease, but it is not known how they cause the disease. Although APP has a receptor-like architecture^{2–5}, is located on the neuronal surface⁶, and has a conserved cytoplasmic domain⁷, no receptor function has been demonstrated for APP. Here we report that APP forms a complex with G_o, a major GTP-binding protein in brain. The cytoplasmic APP sequence His 657–Lys 676 shows a specific G_o-activating function and is necessary for complex formation. G_o protein treated with GTP- γ S lost the ability to associate with APP. This suggests that APP is a receptor coupled to G_o and that abnormal APP–G_o signalling is involved in the Alzheimer's disease process.

We found that the receptor for insulin-like growth factor II (IGF-IIR) couples directly to G_i protein⁸ through a short region, Arg 2,410–Lys 2,423 (refs 9, 10). Recent reports^{11,12} support the concept that short peptides can activate G proteins. We defined the structural determinants for the G_i-activating function in IGF-IIR (ref. 9) as (1) two basic residues at the N-terminal side of the amino-acid sequence and (2) a C-terminal motif of B-B-X-B or B-B-X-X-B (where B is a basic residue, and X is a non-basic residue). To assess whether APP might function as a G-protein-coupled receptor, we searched the amino-acid sequence encoded by the human APP₆₉₅ transcript for regions shorter than 26 residues that satisfy (1) and (2). The sequence His 657–Lys 676 is the only such region in the cytoplasmic domain of APP₆₉₅, and spans from the 9th to the 28th residue after the C-terminal end of the transmembrane domain (Fig. 1a). In two other isoforms of APP, APP₇₅₁ (refs. 3, 4) and APP₇₇₀ (ref. 5), as well as in mouse APP₆₉₅ (ref. 7), this sequence is completely conserved.

The peptide corresponding to this region (referred to as peptide 20) stimulated the rate constant of GTP- γ S binding to G_o in a dose-dependent manner, whereas peptides from other regions of APP₆₉₅ were ineffective (Fig. 1b, c and d). As peptide 20 and peptide_{677–695} almost occupy the full cytoplasmic domain of APP, peptide 20 may be the only cytoplasmic region to have G_o-activator function. Experiments (Fig. 1d) using structural variant peptides indicate that the N-terminal basic residues and the C-terminal B-B-X-X-B motif are essential to the G_o-activating function of peptide 20, suggesting that peptide 20 has an appropriate length for its action. When connected to the 10-residue transmembrane region of APP, peptide 20 became 10–20 times more potent in stimulating vesicle-incorporated G_o.

[†] Present addresses: Department of Medicine, Harvard Medical School, Cardiovascular Research Center, Massachusetts General Hospital-East, 149 13th Street, Charlestown, Massachusetts 02129, USA (I.N., T.O. and T.O.); and Cancer Research Institute, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan (E.O.).

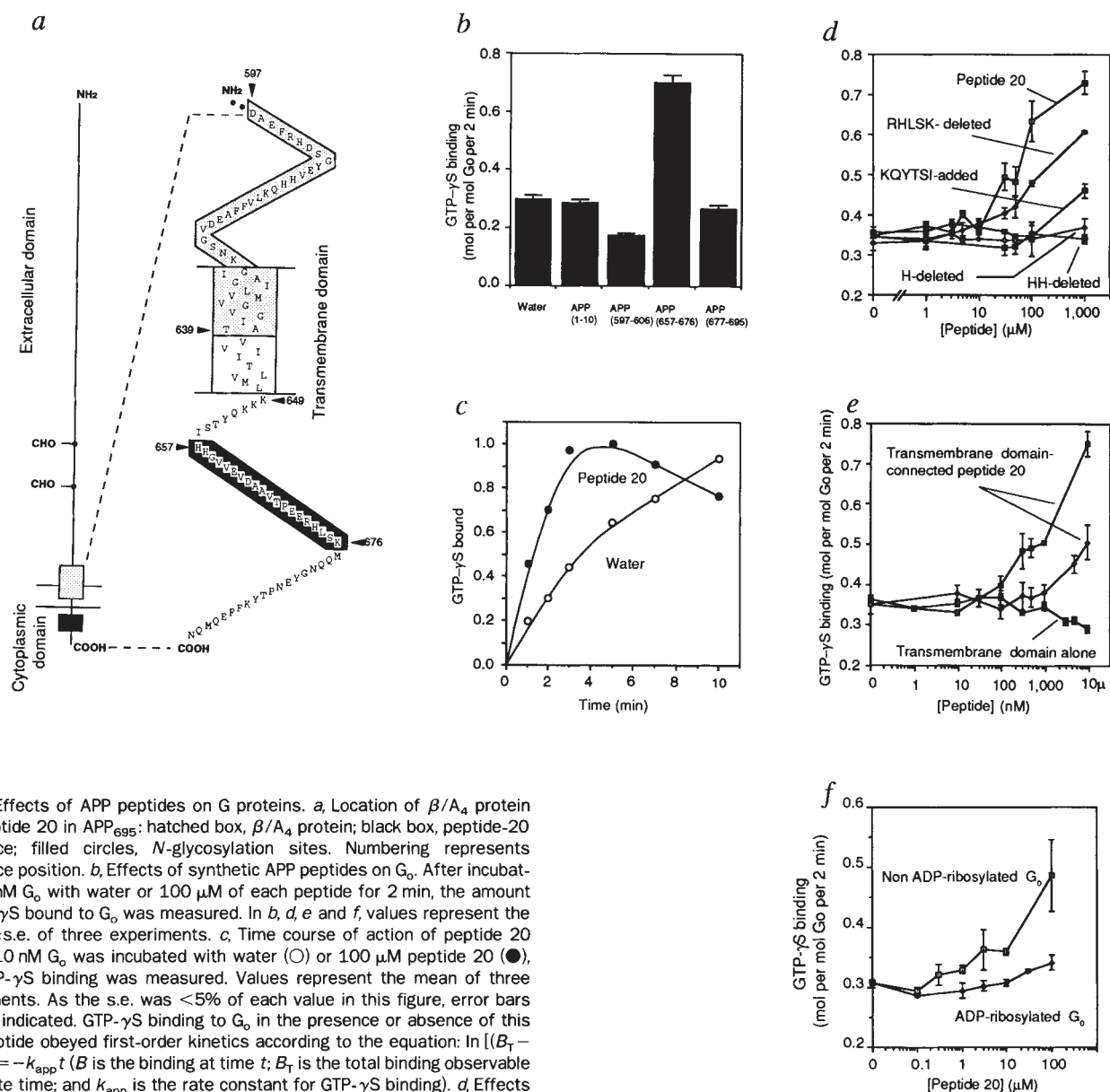


FIG. 1 Effects of APP peptides on G proteins. *a*, Location of β/A_4 protein and peptide 20 in APP₆₉₅: hatched box, β/A_4 protein; black box, peptide-20 sequence; filled circles, N-glycosylation sites. Numbering represents sequence position. *b*, Effects of synthetic APP peptides on G₀. After incubating 10 nM G₀ with water or 100 μM of each peptide for 2 min, the amount of GTP-γS bound to G₀ was measured. In *b*, *d*, *e* and *f*, values represent the mean $\pm$ s.e. of three experiments. *c*, Time course of action of peptide 20 on G₀. 10 nM G₀ was incubated with water (○) or 100 μM peptide 20 (●), and GTP-γS binding was measured. Values represent the mean of three experiments. As the s.e. was <5% of each value in this figure, error bars are not indicated. GTP-γS binding to G₀ in the presence or absence of this APP peptide obeyed first-order kinetics according to the equation: $\ln[(B_t - B)/(B_t - B_0)] = -k_{app}t$ (B is the binding at time t ; B_t is the total binding observable at infinite time; and k_{app} is the rate constant for GTP-γS binding). *d*, Effects of peptide-20 variants on G₀. 10 nM G₀ was incubated with various concentrations of HHGVVEVDAAVTPEERHLSK (peptide 20), HGVVEVDAAVTPEERHLSK (H-deleted), GVVEVDAAVTPEERHLSK (HH-deleted), HHGVVEVDAAVTPEE (RHLSK-deleted), and KQYTSIHGVVEVDAAVTPEERHLSK (KQYTSI-added); and GTP-γS binding to G₀ at 2 min was measured. This figure indicates which aspects of primary structure determine the G₀-activator function of peptide 20. Deletion of either one or both of the N-terminal His residues nullified G₀-activator function of the peptide. The peptide with deletion of the C-terminal RHLSK is several times less potent than peptide 20. KQYTSI is a native sequence adjacent to the peptide-20 sequence in APP. *e*, Effect of connection with a transmembrane region on the action of peptide 20 on G₀. G₀ reconstituted in phospholipid vesicles was incubated with transmembrane region connected peptide 20 (TVIVITLVMLHHGVVEVDAAVTPEERHLSK) or the partial sequence of the APP transmembrane domain alone (TVIVITLVML). Transmembrane region-connected peptide 20 was also incubated with G₀ in the absence of phospholipid and the presence of 0.07% CHAPS (◆). *f*, Effect of pertussis toxin on peptide 20-induced stimulation of GTP-γS binding to G₀. Increasing concentrations of peptide 20 were incubated for 2 min with G₀ reconstituted in phospholipid vesicles which had been treated with pertussis toxin in the presence (◆) or absence (□) of NAD; and GTP-γS binding to G₀ was measured.

METHODS. Synthetic peptides were produced by the solid-phase method, purified by HPLC to >95% purity, and used immediately after synthesis. The ability of peptide 20 to activate G₀ was gradually decreased during storage at either -4 °C or -20 °C. Trimeric G₀ purified from bovine brain was donated by T. Katada²². This G₀ preparation was purified to homogeneity

as described, stored in 20 mM HEPES/NaOH (pH 7.4), 1 mM EDTA and 0.7% CHAPS, and diluted ≥ 10 fold for assays. GTP-γS binding to G₀ was assayed in buffer A (50 mM HEPES/NaOH (pH 7.4), 100 μM EDTA and 120 μM MgCl₂, and 60 nM [³⁵S]GTP-γS) at 37 °C and the fraction bound to GTP-γS of total G₀ was measured as described⁹. The saturated GTP-γS binding to G₀ was determined after 30 min incubation of G₀ with 10 mM Mg²⁺ and GTP-γS of ≥ 60 nM at 30 °C, and defined as the total amount of G₀. This was essentially the same as the GTP-γS binding to G₀ after 60 min incubation of G₀ in buffer A at 37 °C. Thus, the fraction bound to GTP-γS of total G₀ was calculated using the latter value. For experiments exploring the effect of Mg²⁺, the Mg²⁺ concentration was set using Mg-EDTA buffer²³. ADP-ribosylation of G₀ was done by incubating G₀ reconstituted in phospholipid vesicles with 10 μg ml⁻¹ preactivated pertussis toxin in the presence of 10 μM NAD for 15 min at 30 °C as described⁹. Preactivation of pertussis toxin (Funakoshi, Japan) was done by treating the toxin with 100 μM ATP and 1 mM DTT for 10 min at 30 °C. Reconstitution of G₀ into phospholipid vesicles was with 1 mg ml⁻¹ phosphatidylcholine (Sigma; P-5638) at a final G₀ concentration of 50.2 nM in buffer containing 20 mM HEPES/NaOH (pH 7.4), 0.1 mM EDTA, 1 mM DTT and 100 mM NaCl by gel filtration⁹. In a final incubation for GTP-γS binding, 5 nM of reconstituted G₀ was used.

FIG. 2 Precipitation of APP and G_o by an anti-APP antibody from brain membranes. *a*, Immunoprecipitation of APP by 22C11. Solubilized membranes of bovine brain were immunoprecipitated by 22C11 and the pellets were analysed by immunodetection with 22C11 (lanes 1 and 2). Lanes 1 and 3 indicate the controls using no membrane and rabbit IgG for the precipitation, respectively. In both controls, immunodetection was with 22C11. The 55K and 25K bands may be heavy and light chains of 22C11 used for precipitation, which reacted with an anti-mouse IgG antibody during immunodetection. The precipitate by control rabbit IgG contained no detectable APP. Lane 4 indicates the result of immunoblotting of 22C11 precipitate from brain membranes with 1C1, a monoclonal antibody against the C-terminal peptide₆₇₇₋₆₉₅ of APP. Although the size of APP is slightly less than reported, the precipitated form is unlikely to be an extracellular fragment of APP, because 1C1 recognizes this 100K band. Note that the lower bands are at ~25K in lane 4, and are thought to be the light chains of 22C11. *b*, Immunoprecipitation of G_o by 22C11. Precipitation by 22C11 and detection by anti- G protein antibodies. Lane 1, no membrane; lanes 2-6, 22C11 precipitation of brain membranes with immunoblot detection by different antisera (1/1,000 dilution) (lanes 1 and 2: GC/2, anti- $G_{o\alpha}$ antiserum; lane 3: GC/2 plus 1 $\mu\text{g ml}^{-1}$ purified G_o ; lane 4: GA/1, common $G_{o\alpha}$ antiserum; lane 5: AS/7, anti- $G_{o\alpha}$ antiserum; lane 6: MS/1, common $G_{o\beta}$ antiserum). Lane 7 shows the immunodetection with GC/2 of the precipitate by control rabbit IgG from brain membranes. The identity of the 39K protein as G_o was verified by its absence in the 22C11 precipitate unexposed to brain membranes (lane 1), by its staining with another $G_{o\alpha}$ -specific antibody $\alpha G01$ (ref. 24) (not shown) and by a blockade of staining in the presence of excess soluble G_o (lane 3). The 22C11 precipitate also contained immunoreactivity of $G_{o\beta}$ in a doublet at 35-36K (lane 6). The 22C11 precipitate did not react with an anti- $G_{o\alpha}$ antibody AS/7 (lane 5). The antibody GA/1 detected only a 39K band in the 22C11 precipitate (lane 4). The precipitate by control rabbit IgG contained neither immunoreactivity of APP nor G_o (lane 7). *c*, Effect of Mg^{2+} on the immunoprecipitation of G_o by 22C11. 100 μg of solubilized brain membranes were precipitated by 22C11 in the presence of various Mg^{2+} concentrations set with Mg -EDTA buffer²³. Precipitates were analysed with immunoblot by GC/2. Control lane indicates the precipitation of brain membranes by rabbit IgG and the immunodetection by GC/2. In the absence of Mg^{2+} , G_o was less efficiently precipitated by 22C11. Mg^{2+} concentrations between 1 μM and 1 mM resulted in maximal immunoprecipitation of G_o . At the concentrations >10 mM, G_o was scarcely precipitated. *d*, Effect of peptide 20 on 22C11-induced precipitation of $G_{o\alpha}$ (left) and APP (right). Solubilized brain membranes were incubated with 22C11-coated beads in the presence of 10 μM peptide 20 (2nd and 5th lanes) or 10 μM peptide₆₇₇₋₆₉₅ of APP (3rd and 6th lanes) or in the absence of these peptides (1st and 4th lanes). In this experiment, an anti-mouse IgG antibody different from that used in *a* was used.

METHODS. Bovine brain membranes, prepared as described²² and suspended in buffer B (10 mM HEPES/NaOH (pH 7.4), 1 mM EDTA, 10 mM ascorbic acid, 250 mM sucrose, and a mixture of 2 mM PMSF, 20 $\mu\text{g ml}^{-1}$ aprotinin, and 20 μM leupeptin (PAL)), were centrifuged and the pellet solubilized for 1 h at 4 °C with buffer C (10 mM HEPES/NaOH (pH 7.4), 1 mM EDTA, 120 mM NaCl, 0.5% CHAPS and PAL). The material was centrifuged at 15,000 r.p.m. for 1 h. The supernatant (500 μg protein unless specified) was incubated in buffer D (20 mM HEPES/NaOH (pH 7.4), 1 mM EDTA, 120 mM NaCl and PAL) and 2% BSA with 22C11-coated protein G-Sepharose, which had been prepared by incubating protein G-Sepharose (Pharmacia) with 22C11 (Boehringer Mannheim) for 1 h at 4 °C. The final concentration of 22C11 was 2 $\mu\text{g ml}^{-1}$. As a control, 2 $\mu\text{g ml}^{-1}$ of rabbit IgG was used. After overnight shaking at 4 °C, the sample was centrifuged at 5,000 r.p.m. for 5 min. The pellet was washed three times with ice-cold buffer D and the final pellet was applied to SDS-PAGE. Electrophoresis onto a PVDF sheet was as described²⁵. After blocking with PBS containing 2% skim milk and 1% BSA, the sheet was incubated with the first antibody (1 $\mu\text{g ml}^{-1}$ 22C11; 1/1,000 dilution of GC/2; 1/1,000 dilution of 1C1) for 4 h, and then exposed to HRP-conjugated goat IgG reactive for mouse or rabbit immunoglobulin for 2-4 h at room temperature. Antigenic bands were detected with an ECL detection kit (Amersham). Anti- G protein antibodies were from New England Nuclear. YL1/2 (SERA Lab), an anti-tubulin antibody, was provided by E. Nishida and used at 1/500 dilution for immunodetection. Each result was reproduced at least three times.

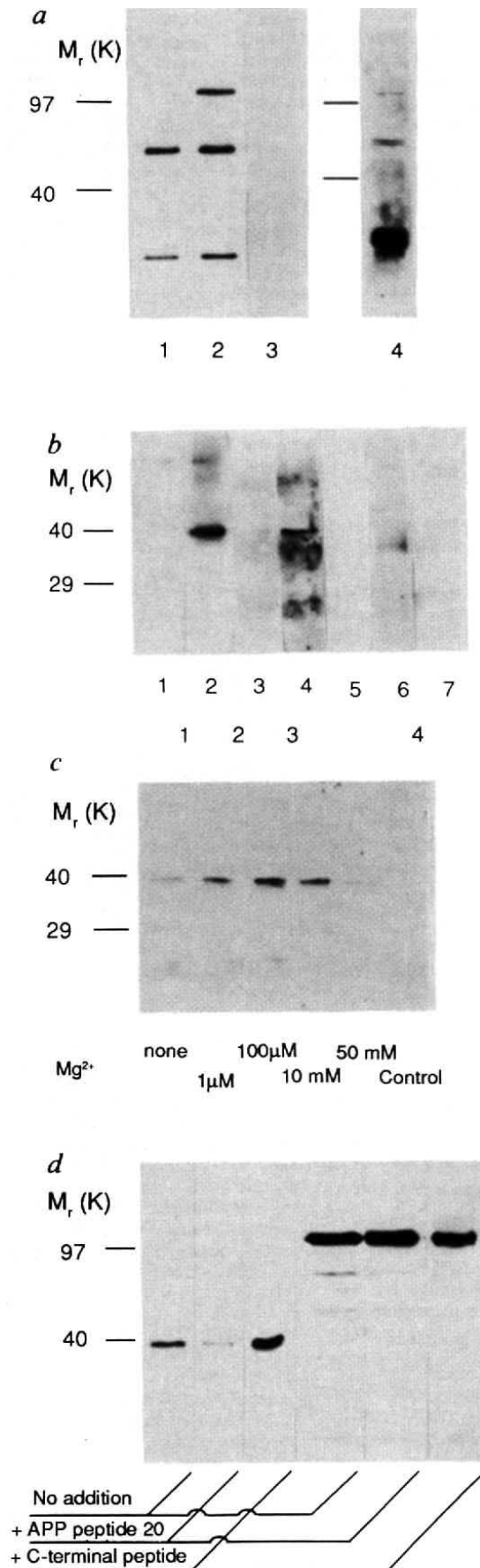


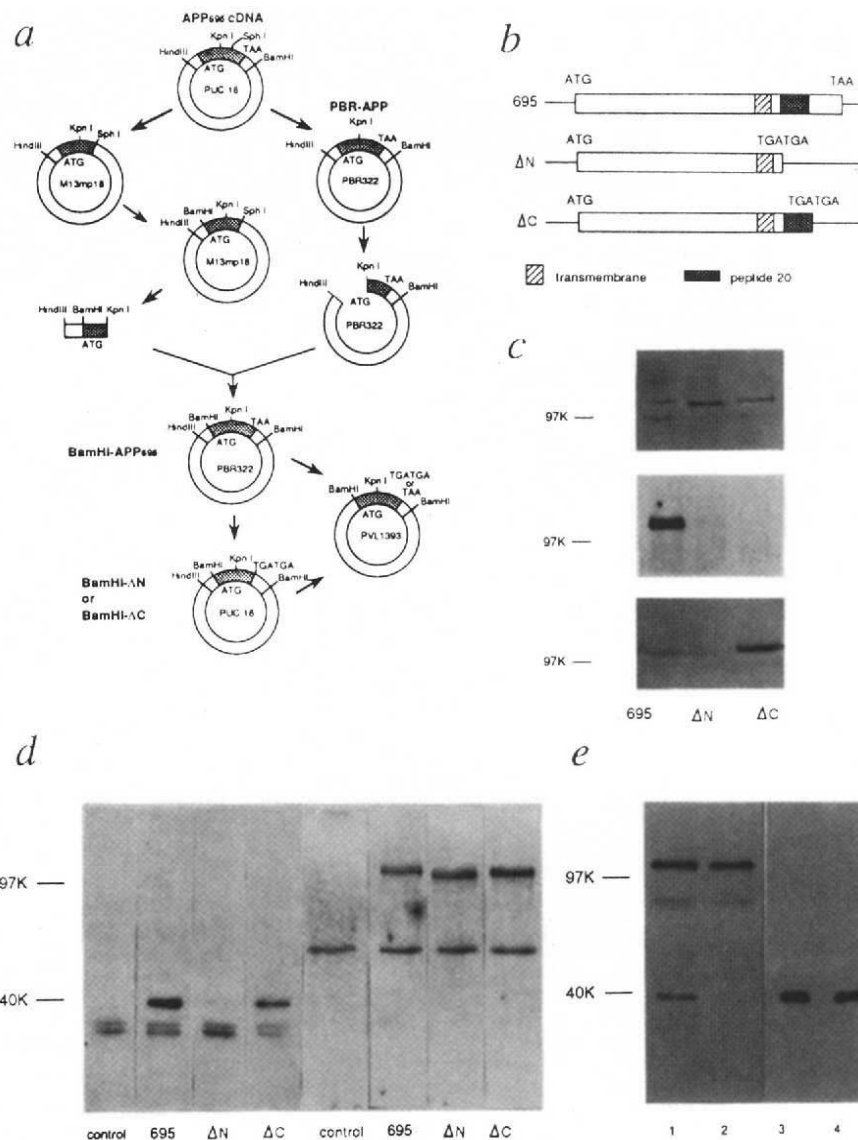
FIG. 3 Precipitation of G_0 reconstituted with recombinant APP-antibody complex. *a*, Construction method of mutant APP cDNAs. *b*, Schematic illustration of construction of authentic APP₆₉₅ and mutant APP cDNAs. Regions labelled ATG, TAA, TGA signify original initiation and termination sites and a newly inserted termination site, respectively. *c*, Immunoblot analysis by anti-Alz 90, 1C1, and 4G5 of Sf9 membranes. Sf9 membranes (left: Sf9-APP₆₉₅; middle: Sf9-ΔN; right: Sf9-ΔC) were analysed for immunoreactivity for anti-Alz 90 (upper), 1C1 (middle), and 4G5 (bottom). Anti-Alz 90 (Boehringer Mannheim), 1C1 and 4G5 are mouse monoclonal antibodies against residues 511–608 (extracellular domain), 677–695 (C-terminal portion of the cytoplasmic domain), and 657–676 (peptide-20 region of the cytoplasmic domain) of APP, respectively. 1C1 and 4G5 were raised originally by us. Anti-Alz 90 recognized 130K, 120K, and 130K proteins in Sf9-APP₆₉₅, –ΔN, and –ΔC cells, respectively; 1C1 recognized the 130K band in Sf9-APP₆₉₅ but no proteins in Sf9-ΔN or –ΔC; 4G5 reacted with the 130K band in Sf9-APP₆₉₅ and –ΔC, but not with the 120K band in Sf9-ΔN. The Sf9-APP₆₉₅ cells expressed APP at ~0.1% of the total membrane protein. *d*, Immunoblot analysis of the 22C11 precipitate from the Sf9 membrane- G_0 reconstitution mixture. The 22C11 precipitate from Sf9 membranes expressing various forms of APP was further incubated with purified G_0 , then the final precipitate was analysed by immunoblotting with an anti- $G_{0\alpha}$ antibody αG01 (left four lanes) and by 22C11 (right four lanes). The control lane indicates the 22C11 precipitate exposed to G_0 in the absence of Sf9 membranes. The same sample was loaded in each pair of lanes. αG01 (ref. 24) was provided by T. Asano. Similar results were obtained using GC/2. *e*, Dissociation of G_0 from APP by activation. The 22C11 precipitates from Sf9-APP₆₉₅ membranes (100 μg protein) were incubated with activated G_0 (lanes 2 and 4) or unactivated G_0 (lanes 1 and 3), and the final precipitates (left panel) and supernatants (right panel) were analysed by simultaneous detection with 22C11 and αG01. Activation of G_0 was achieved by incubating G_0 in 20 mM HEPES/NaOH (pH 7.4), 1 mM EDTA, 2 mM MgCl₂ and 1 μM GTP-γS overnight at room temperature.

METHODS. The outline of the mutant APP construction is indicated in *a*. Authentic mouse APP₆₉₅ cDNA was provided by Y. Sakaki⁷ in the vector pUC18. The *Hind*III–*Bam*HI fragment containing the entire coding region was initially subcloned into the vector pBR322 (pBR-APP). A single *Bam*HI site was inserted immediately before the ATG codon of the *Hind*III–*Sph*I fragment. This *Bam*HI site was inserted to express the coding APP proteins efficiently in baculovirus-infected cells. The *Bam*HI site-inserted APP₆₉₅-coding DNA (*Bam*HI-APP₆₉₅) was constructed from the *Hind*III–*Sph*I fragment and pBR-APP using their internal *Kpn*I sites and subcloned into pUC18. By using *Bam*HI-APP₆₉₅ as a template, two truncation mutants were generated and subcloned into pUC18. They have an insertion of two TGA codons immediately before (ΔN) and after (ΔC) the peptide-20 sequence. Each *Bam*HI–*Bam*HI fragment of these pUC18 plasmids encoding APP cDNAs was constructed into the baculovirus transfer/expression vector pVL1393 (Invitrogen). The entire region that had been through a single-stranded intermediate was sequenced to confirm the absence of unwanted nucleotide changes. New insertions were generated by oligonucleotide-directed mutagenesis with a kit (Takara)²⁶. For insertion of a *Bam*HI site, a restriction fragment encoding the ATG start codon was subcloned into the vector M13mp18, and a single-stranded template was generated. An oligonucleotide primer (CCACG-CAGGATACGGGATCCATGCTGCCAGCTTG) was used to introduce GGATCC immediately before the start codon. After primer extension, the phage was used to transform *E. coli* strain JM109. Plaques were selected and single-

stranded DNA was sequenced. A restriction fragment containing the mutated region was subcloned into pBR-APP. For the insertion of the stop codons, oligonucleotide primers (CAGTACATCCATCTGATGACATCATGGCGTGGTG and CGCCATCTCTCCAAGTGATGAATGCAGCAGAACGGA) and the M13mp19 vector were used to introduce two sequential TGA stop codons. According to (ref. 27), baculoviruses encoding these APP cDNAs were generated by selection using immunoblot analysis with 22C11 and recovered by infecting Sf9 cells. Four days after treatment of Sf9 cells with the viruses, cells were homogenized and suspended in buffer B. After solubilization of the pellet with buffer C, the supernatant (100 μg) was mixed overnight with 22C11-coated protein G-Sepharose in buffer D plus 2% BSA at 4 °C on a shaker. After centrifugation, precipitated beads were incubated with purified G_0 (1 μg) in buffer D supplemented with 1.1 mM MgCl₂ and 2% BSA for 8–24 h at 4 °C on a shaker. After washing four times with ice-cold buffer D, the centrifuged precipitate was subjected to SDS-PAGE, electroblotting and immunodetection with the first antibodies (1 μg ml^{−1} 22C11; 10 μg ml^{−1} anti-Alz 90; 1/1,000 dilution of 1C1; 1/500 dilution of 4G5; 0.1 μg ml^{−1} αG01) and the second goat anti-mouse or anti-rabbit IgG conjugated with HRP as already described. For the second antibody against 1C1 and 4G5, both of which belong to mouse IgM (κ), the mixture of the HRP-conjugated anti-rabbit IgG with rabbit anti-mouse IgM and anti-κ antibodies was used. Each of the results was reproduced at least three times.

without altering its efficacy (Fig. 1*e*), suggesting that the peptide 20 sequence included in APP is more potent than synthetic peptide 20. The effect of peptide 20 could not be observed at low (≤100 nM) or high Mg²⁺ concentrations (≥10 mM). In contrast, peptide 20 produced 2–3-fold stimulation of GTP-γS

binding to G_0 in the mid-range of Mg²⁺ concentrations (100 nM–1 mM). Peptide 20 had little effect on other G proteins; G_{i1} , G_{i2} , G_{i3} , G_s , c-Ki-ras p21 and smg p25A (G_{i1} , G_{i2} , and G_s donated by T. Katada, G_{i3} by T. Asano, and c-Ki-ras p21 and smg p25A by Y. Takai) were not stimulated (data not shown).



The peptide 20 action on G_o was significantly attenuated when G_o had been treated with pertussis toxin in the presence of NAD (Fig. 1f), whereas peptide 20 action was weaker but still significant on the control G_o pretreated with the toxin in the absence of NAD (the weaker action may be attributed to heat denaturation of G_o). It has been reported that G_o activation by GAP-43 is insensitive to pertussis toxin¹³. Thus, GAP-43 and peptide 20 may recognize the different sites on $G_{o\alpha}$.

We therefore considered that APP might interact directly with G_o through the peptide 20 region. We investigated whether APP was linked to G_o in a native membrane environment. Our strategy was to precipitate proteins in brain membranes by the monoclonal anti-APP antibody 22C11 and examine the precipitate by immunoblotting with anti-G-protein antibodies. The 22C11 precipitate from brain membranes contained APP immunoreactivity at a size corresponding to M_r 100,000 (100K) (Fig. 2a). The same 22C11 precipitate was found to contain $G_{o\alpha}$ immunoreactivity at 39K and $G_{o\beta}$ immunoreactivity in a doublet at 35–36K, but no detectable $G_{i\alpha}$ or other heterotrimeric G proteins (Fig. 2b). A tubulin antibody, YL1/2, did not stain the 22C11 precipitate (data not shown).

The concentration of Mg^{2+} drastically altered the 22C11-mediated precipitation of G_o (Fig. 2c), whereas the immunoprecipitation of APP was not affected by Mg^{2+} (data not shown). Mg^{2+} was not absolutely required for complex formation of APP with G_o , and a mid-range Mg^{2+} concentration facilitated APP- G_o association. Peptide 20 prevented the 22C11-mediated precipitation of G_o , whereas it did not affect the precipitation of APP by 22C11 (Fig. 2d). The control peptide, peptide_{677–695} of APP, had no inhibitory effect on 22C11-mediated precipitation of G_o or APP.

Sf9 cells were infected by recombinant baculoviruses encoding full-length APP₆₉₅ complementary DNA, APP_{1–656} cDNA (ΔN), or APP_{1–676} cDNA (ΔC) (Fig. 3a and b). The ΔC deletion retains the peptide 20 region and the ΔN deletion lacks it. In uninfected Sf9 cells, no immunoreactivity for APP or G_o was detected (data not shown). The membranes of Sf9 cells infected with the viruses encoding APP₆₉₅, ΔN or ΔC genes (referred to as Sf9-APP₆₉₅, Sf9- ΔN and Sf9- ΔC) were found to express, respectively, 130K, 120K and 130K proteins reactive with antibody 22C11 (Fig. 3d). Experiments using other anti-APP antibodies (Fig. 3c) clearly indicate that these proteins are the recombinant APP_{1–695}, APP_{1–656} and APP_{1–676}, respectively, as designed. There was no difference in the amount of precipitated APP among these Sf9 membranes.

The 22C11 precipitate from these Sf9 membranes was exposed to purified G_o , then the second precipitate was subjected to immunoblot analysis. Immunoreactive $G_{o\alpha}$ was observed at 39K in the 22C11 precipitate from Sf9-APP₆₉₅ membranes exposed to G_o (Fig. 3d). When G_o was incubated with GTP- γ S, no $G_{o\alpha}$ associated with the APP-22C11 complex (Fig. 3e), suggesting that the G-protein activation state regulates APP- G_o association. About 1/10–1/20 (0.05–0.1 μ g per tube) of the reconstituted G_o was precipitated, together with a comparable amount ($\sim$ 0.1 μ g per tube) of APP. Easily detectable amounts of $G_{o\alpha}$ were present in the final precipitate when G_o was mixed with 22C11 precipitates from Sf9- ΔC or Sf9-APP₆₉₅ membranes, but essentially no $G_{o\alpha}$ was found in the final precipitate from Sf9- ΔN membranes. Thus, formation of an APP- G_o complex requires the peptide 20 region, residues 657–676.

This study provides evidence that APP functions as a receptor coupled to G_o through the G_o -activator cytoplasmic domain His 657–Lys 676. APP has a point mutation in the familial form of Alzheimer's disease¹⁴. A structural alteration of APP is therefore thought to be one cause of Alzheimer's disease, although how it might do so remains unknown. One possibility suggested by our results could be that the cytoplasmic C-terminal fragment of APP is pathogenic. It has been suggested^{15–17} that the residual C-terminal portion of APP may remain in the cell membrane after abnormal cleavage of APP into β/A_4 protein in Alzheimer's

disease neurons. By analogy with the oncogenic transformation of c-erbB into v-erbB, such a structural alteration of APP may alter its function and prompt APP to activate G_o constitutively. This hypothesis is consistent with results¹⁸ indicating that expression of the C-terminal 105-residue portion of APP evokes neuronal death, and with reports that G_o activity is linked to neuronal growth cone motility¹⁹, axon and dendrite formation²⁰, and memory²¹. This study opens the possibility that Alzheimer's disease is a disorder of an APP- G_o signalling system caused by structural alterations of APP. □

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Cell-free activation of a DNA-binding protein by epidermal growth factor

Henry B. Sadowski & Michael Z. Gilman*

Cold Spring Harbor Laboratory, Box 100, 1 Bungtown Road, Cold Spring Harbor, New York 11724-2206, USA

GROWTH factors such as platelet-derived growth factor and epidermal growth factor (EGF) bind to and activate cell-surface receptors with intrinsic tyrosine kinase activities¹. Receptor activation elicits multiple physiological changes in target cells, including alterations in gene expression^{2–4}. Receptor tyrosine kinase signalling involves recruitment of proteins into a signalling complex through interactions between receptor autophosphorylation sites and the src-homology region-2 (SH2) domains on these signalling proteins^{5–9}. Diverse signals can subsequently be generated, depending on the specific receptor and cell type^{2,10}. How such signals are transmitted to the nucleus is poorly understood, but because the transcriptional activation of many genes by growth factors occurs in the absence of new protein synthesis¹, one or more signals emanating from growth factor receptors must directly affect

* To whom correspondence should be addressed

transcription factors. We report here the activation by EGF of a DNA-binding protein in a cell-free system where activation of DNA binding requires ligand, receptor, ATP and phosphotyrosine-SH2 interactions.

Transcription of the *c-fos* proto-oncogene is rapidly activated by polypeptide growth factors¹¹ through protein kinase C-dependent and -independent pathways¹²⁻¹⁴. Maximal activation requires the serum response element (SRE), located 300

base pairs (bp) upstream of the transcription start site¹⁵. But an additional element, the SIE (*c-sis* inducible element), located about 25 bp 5' of the SRE, cooperates with other sites in the *c-fos* promoter to mediate induction by platelet-derived growth factor (PDGF) in fibroblasts¹⁶. This element binds a protein, termed SIF (*c-sis* inducible factor), whose latent DNA-binding activity is induced within minutes after treatment of BALB/c 3T3 cells with PDGF¹⁷. Genomic footprinting experiments

FIG. 1 Activation of SIF DNA-binding activity by EGF in A431 cells. Mobility-shift assays of SIE (lanes 1-8) or SRE (lanes 9-16) binding proteins in cytoplasmic (lanes 5-8 and 13-16) or nuclear (lanes 1-4 and 9-12) fractions (15 µg protein) from A431 cells treated with EGF (100 ng ml⁻¹) for the indicated times (in s).

METHODS. A431 cells (10 cm dishes) were grown to confluence in DMEM with 10% fetal bovine serum (FBS), then serum-starved for 48 h (0.5% FBS). After treatment with EGF, cells were rinsed twice with ice-cold PBS, once with PBS containing 1 mM Na₃VO₄ and 5 mM NaF (PBS + V + F), and once with hypotonic buffer. Ice-cold hypotonic buffer with 0.2% Nonidet P-40 (NP-40) (0.5 ml) was added directly to the dishes to lyse cells. Lysates were scraped into microfuge tubes and mixed, and the nuclei pelleted (16,000g for 20 s). Supernatants were supplemented with NaCl to 120 mM, clarified (16,000g for 20 min), and glycerol was added to 10% ('cytosol'). The nuclear pellets were resuspended in 150 µl of high salt buffer and gently rocked for 30 min. Extracted proteins were separated from residual nuclei (16,000g for 20 min) ('nucleus'). Hypotonic buffer consisted of: 20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM Na₃VO₄, 1 mM Na₂P₂O₇, 0.125 µM okadaic acid or 0.4 µM microcystin, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 1 µg ml⁻¹ each leupeptin, aprotinin and pepstatin. This buffer containing 420 mM NaCl and 20% glycerol (high salt buffer) was used for nuclear extraction. For mobility-shift assays, cytoplasmic or nuclear extracts were incubated with poly(dI-dC) poly(dI-dC) in binding buffer for 15 min on ice, after which ³²P-labelled oligonucleotide SIE (mutant 67 in ref. 16) or SRE (ref. 28) probes (20,000 c.p.m., ~5 fmol) were added and the reactions incubated for 15 min at 25 °C. Final binding reactions (20 µl) contained: 13 mM HEPES, pH 7.9, 65 mM NaCl, 1 mM DTT, 0.15 mM EDTA, 8% glycerol, 50 µg ml⁻¹ poly(dI-dC):poly(dI-dC) and 0-0.02% NP-40. Reactions were analysed on 5% polyacrylamide gels (39:1 acrylamide:bis) containing 2.5% glycerol, in 0.5× TBE (Tris/borate/EDTA) buffer. SRE-binding proteins in lanes 9-16 are, from the bottom, p62^{DBF}, SRF and SRF-TCF ternary complex¹⁵.

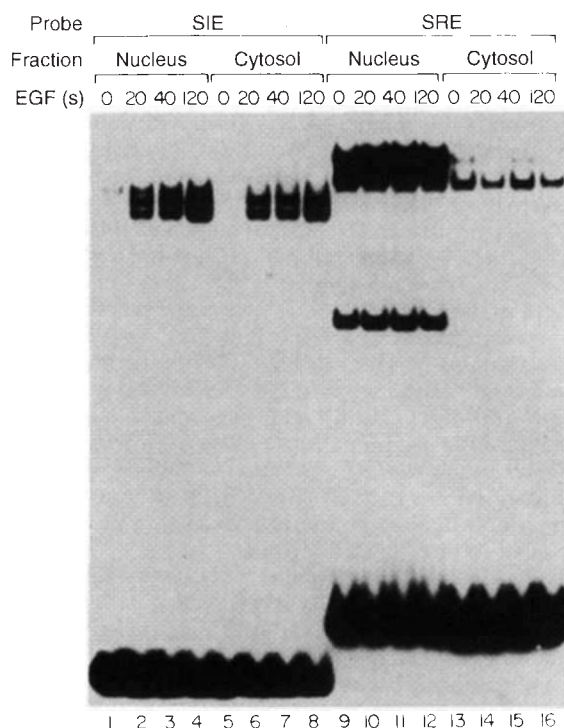
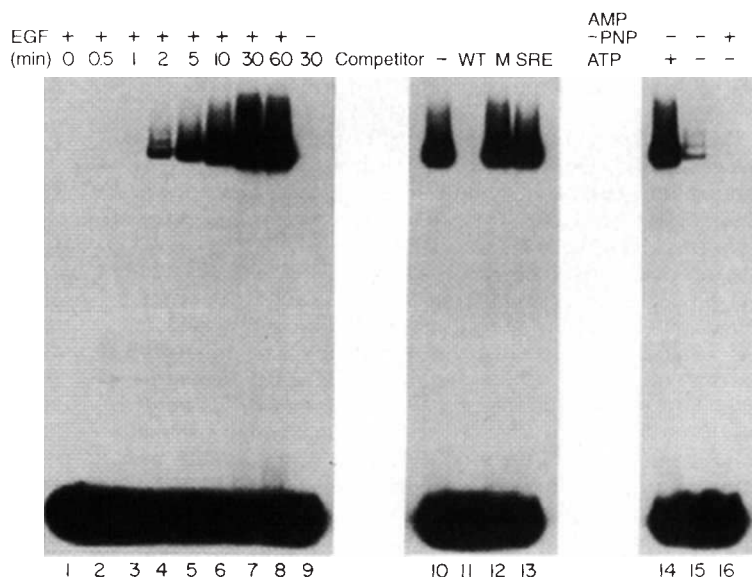


FIG. 2 Cell-free activation of SIF DNA-binding activity by EGF. Reactions (20 µl) containing membrane, cytoplasmic and nuclear fractions from unstimulated A431 cells were incubated for 15 min on ice in the absence (lane 9) or presence of 2 µg ml⁻¹ EGF (lanes 1-8 and 10-16), after which buffer (5 µl) containing ATP (lanes 1-14), no ATP (lane 15), or AMP-PNP (lane 16) was added, and the reactions incubated at 30 °C for the indicated times (in min). Reactions in lanes 9-16 were incubated for 30 min. Reactions were quenched on ice with EDTA (13.5 mM), briefly centrifuged, and aliquots subjected to SIE mobility-shift assays, in the absence (lanes 1-10 and 14-16) or presence of 100-fold molar excess of unlabelled competitor oligonucleotides.

METHODS. Confluent dishes of A431 cells (20 15-cm plates, ~1.5 × 10⁹ cells) were rinsed with ice-cold PBS, scraped from the dishes, and pelleted. Cytoplasmic and nuclear high-salt extract fractions were prepared essentially as described²⁹ with the following modifications. Hypotonic and high-salt buffers (Fig. 1), supplemented with 0.4 mM ammonium molybdate, were substituted for buffers A and C. The cells were lysed in three packed-cell volumes (PCV) of hypotonic buffer with 20 strokes of a type A pestle. Crude nuclei were extracted with the high salt buffer, and the nuclear extract was frozen without dialysis. The low-speed supernatant was adjusted to 120 mM NaCl and centrifuged for 60 min. at 100,000g. Glycerol was added to the high-speed supernatant to 10% and this cytosolic fraction was frozen without dialysis. The high-speed pellet was resuspended in 2 PCV of hypotonic buffer containing 150 mM NaCl and 8% glycerol, centrifuged (16,000g for 30 min), and the membrane pellets frozen. Membranes (~600 µg protein) were thawed on ice, resuspended in 90 µl MRB, and 80 µl of resuspended membranes were added to 80 µl of MRB with 0.5% Triton X-100. MRB consisted of 20 mM Tris, pH 7.4, 20 mM NaF, 0.1 mM Na₃VO₄,



1 mM EDTA, 10% glycerol, 1 µg ml⁻¹ each of leupeptin, aprotinin and pepstatin, with 1 mM DTT and 0.5 mM PMSF added just before use. Detergent-treated membrane fraction (6 µl) was added to tubes containing both cytosolic (8 µl) and nuclear (5 µl) fractions (~20 µg protein of each fraction), and the activation reactions done as described above. Kinase buffer contained 50 mM HEPES, pH 7.4, 20 mM MgCl₂, 10 mM MnCl₂ and either 10 mM ATP, no ATP or 10 mM AMP-PNP. Mobility-shift binding reactions contained 2% Ficoll 400 and 4% glycerol instead of 8% glycerol.

suggested that an SIE-binding activity was also induced by EGF in A431 cells¹⁸. To confirm that authentic SIF activity is induced by EGF in A431 cells, we prepared nuclear and cytoplasmic fractions from these cells and did mobility-shift analysis with a high-affinity SIF binding site¹⁶. Figure 1 shows that SIE binding activity was detectable in both nuclear and cytoplasmic fractions within 20 s of treatment with EGF (lanes 1–8). Peak activity was reached within 2 min and persisted for at least 60 min (data not shown). The activity was sequence-specific and comigrated with authentic SIF activity from PDGF-treated BALB/c 3T3 cells (data not shown, but see Figs 2 and 3b). In contrast to SIF, protein binding to the *c-fos* SRE remained relatively constant after EGF treatment (lanes 9–16). SIF activity was detected at similar levels in nuclear and cytoplasmic fractions (lanes 1–4

versus 5–8), whereas SRE-binding proteins partitioned nearly completely into the nuclear fraction (lanes 9–12 versus 13–16). We believe that the abundance of SIF in the cytoplasmic fraction is significant, but we cannot rule out that it leaked from nuclei after cell lysis.

The level of SIF activity in EGF-treated A431 cells was much higher than we had observed in other cell lines, perhaps due to the high number of EGF receptors in these cells¹⁹. Consequently, we investigated whether SIF activation could be observed in cell-free extracts prepared from unstimulated A431 cells. Cells were fractionated into membrane, cytosol, and nuclear fractions. Cytosol and nuclear fractions were mixed with detergent-treated membrane fraction and incubated with EGF on ice for 15 min. After EGF pretreatment, ATP and divalent cations were added

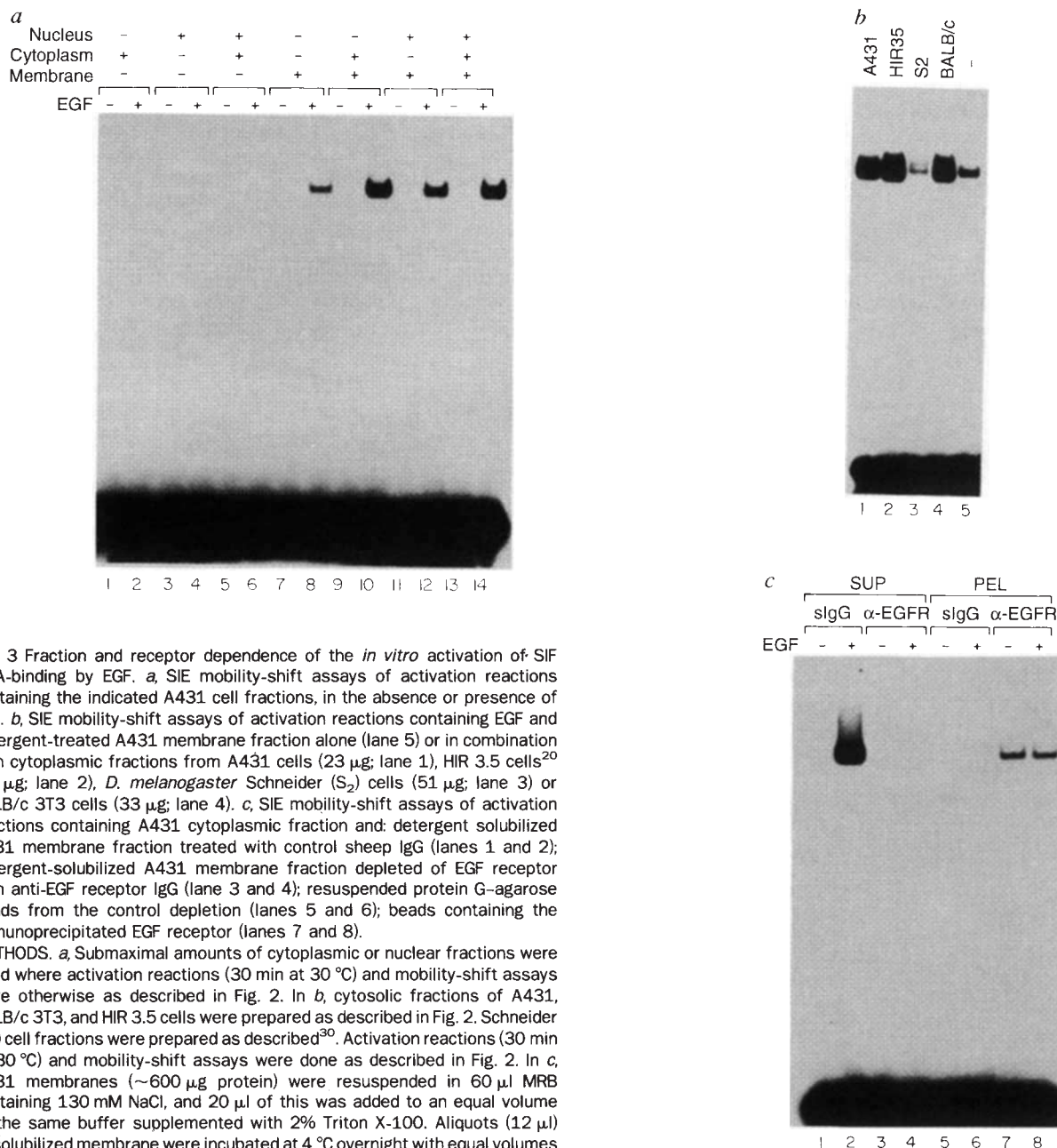


FIG. 3 Fraction and receptor dependence of the *in vitro* activation of SIF DNA-binding by EGF. **a**, SIE mobility-shift assays of activation reactions containing the indicated A431 cell fractions, in the absence or presence of EGF. **b**, SIE mobility-shift assays of activation reactions containing EGF and detergent-treated A431 membrane fraction alone (lane 5) or in combination with cytoplasmic fractions from A431 cells (23 µg; lane 1), HIR 3.5 cells²⁰ (36 µg; lane 2), *D. melanogaster* Schneider (*S*₂) cells (51 µg; lane 3) or BALB/c 3T3 cells (33 µg; lane 4). **c**, SIE mobility-shift assays of activation reactions containing A431 cytoplasmic fraction and: detergent solubilized A431 membrane fraction treated with control sheep IgG (lanes 1 and 2); detergent-solubilized A431 membrane fraction depleted of EGF receptor with anti-EGF receptor IgG (lane 3 and 4); resuspended protein G-agarose beads from the control depletion (lanes 5 and 6); beads containing the immunoprecipitated EGF receptor (lanes 7 and 8).

METHODS. **a**, Submaximal amounts of cytoplasmic or nuclear fractions were used where activation reactions (30 min at 30 °C) and mobility-shift assays were otherwise as described in Fig. 2. In **b**, cytosolic fractions of A431, BALB/c 3T3, and HIR 3.5 cells were prepared as described in Fig. 2. Schneider (*S*₂) cell fractions were prepared as described²⁰. Activation reactions (30 min at 30 °C) and mobility-shift assays were done as described in Fig. 2. In **c**, A431 membranes (~600 µg protein) were resuspended in 60 µl MRB containing 130 mM NaCl, and 20 µl of this was added to an equal volume of the same buffer supplemented with 2% Triton X-100. Aliquots (12 µl) of solubilized membrane were incubated at 4 °C overnight with equal volumes of normal sheep IgG (2 mg ml⁻¹ in PBS) or sheep anti-EGFR IgG (2 mg ml⁻¹ in PBS) (obtained from UBI). Protein-G agarose (12 µl of a 50:50 slurry in MRB containing 130 mM NaCl and 0.5% Triton X-100) were added to each tube and immune complexes collected by gentle rocking for 2 h at 4 °C. The beads were pelleted (16,000g for 15 s), supernatants removed, and aliquots of each supernatant were assayed in activation reactions with A431 cyto-

plasmic fraction. The pellets were washed three times with MRB 130 containing 0.5% Triton X-100, resuspended in the same buffer and aliquots of each were assayed in activation reactions with A431 cytoplasmic fraction. Activation reactions (30 min at 30 °C) and mobility-shift assays were done as in Fig. 2.

and incubation continued for various lengths of time at 30 °C. After quenching with EDTA, aliquots of the reactions were subjected to mobility-shift assay. Figure 2 shows that SIF activity was generated in these reactions in a time-dependent fashion (lanes 1–8), with peak activity reached at about 30 min. Activation was rapid, detectable as early as 30 s on longer exposures of the gel, and not observed in the absence of EGF (lane 9). That this activity is authentic SIF was demonstrated in a competition assay (lanes 10–13). Activity was completed by a high-affinity SIF binding site but not by a biologically inactive mutant¹⁶ nor by the unrelated SRE. Activation of SIF was largely dependent on the addition of ATP. Omission of ATP resulted in a significant reduction of SIF activation (lane 15), and addition of a non-hydrolysable ATP analogue inhibited activation further (lane 16). SIF activation was accompanied by an increase in phosphotyrosine in the EGF receptor and other proteins in the reaction (data not shown). We infer that SIF activation requires ATP hydrolysis, consistent with a requirement for, at a minimum, the protein-tyrosine kinase activity of the EGF receptor.

Figure 3a shows that SIF activation required detergent-treated A431 membrane fraction (compare lanes 6 and 14). Membranes alone generated low levels of SIF activity in the presence of EGF (lane 8), but activity was greatly augmented by the inclusion of either cytosol (lane 10) or to a lesser extent nuclear extract (lane 12). The combination of both cytosol and nuclear extract with membranes led to at most an additive induction of SIF activity (lane 14), suggesting that latent SIF activity and any other activators were present in both fractions, although on

a per cell basis, latent SIF was greatly enriched in cytosol. Cytosol from other cell lines that contain latent SIF activity could substitute for A431 cytosol (Fig. 3b, lanes 2 and 4), whereas cytosol from a *Drosophila* embryo cell line that did not contain latent SIF activity was negative in the *in vitro* assay (lane 3). Despite the fact that SIF can be induced by PDGF in BALB/c 3T3 cells and by insulin in HIR 3.5 cells²⁰ (an NIH 3T3 cell line expressing the human insulin receptor), only detergent-treated membranes from A431 cells supported significant SIF formation *in vitro* (data not shown), suggesting that a rate-limiting component of the activation reaction, possibly the receptor, is particularly abundant in A431 cells.

To demonstrate that activation required the EGF receptor, solubilized A431 membranes were depleted of receptor with polyclonal anti-receptor antibody. Membranes mock-depleted with control antibodies supported EGF-dependent SIF activation (Fig. 3c, lanes 1 and 2), whereas membranes depleted with receptor antibody did not (lanes 3 and 4). Thus, EGF receptor (and/or associated proteins) is required for SIF activation. When the EGF-R antibody-bound material was added to A431 cytosol, SIF activation was observed (lanes 7 and 8), although at significantly lower levels than mock-depleted membrane fractions (compare lanes 8 and 2) and in a ligand-independent manner. Material bound to the control antibody did not support SIF activation (lanes 5 and 6). Thus, immunopurified receptor (and/or associated proteins) can partially substitute for an A431 membrane fraction.

To address whether phosphotyrosine residues on the EGF receptor or other proteins were required for SIF activation, we

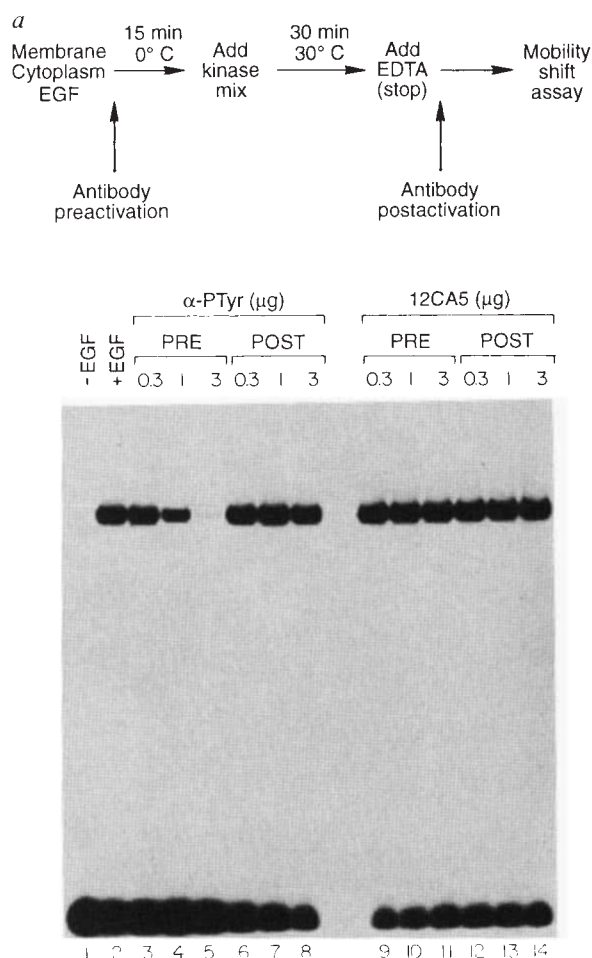
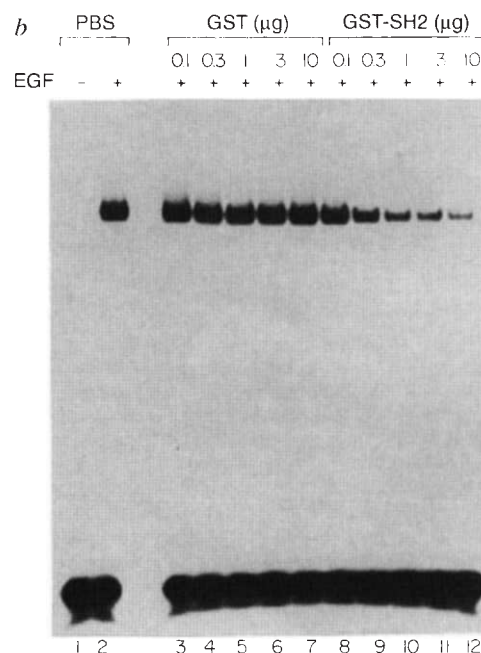


FIG. 4 *In vitro* activation of SIF DNA-binding activity requires phosphotyrosine-SH2 domain interactions. *a*, SIE mobility-shift assays of activation reactions where antibodies were added at the beginning of the reaction



or after the 30-min incubation at 30 °C. Reactions were assembled on ice in the absence (lane 1) or presence (lanes 2–14) of EGF. Indicated amounts of monoclonal antiphosphotyrosine antibody 4G10 (ref. 21, obtained from UBI) or an irrelevant monoclonal antibody (12CA5, ref. 22) in 3 μl PBS were added either before or after activation. When antibody was not added, 3 μl of PBS was substituted. *b*, Mobility-shift assays of activation reactions done in the absence (lane 1) or presence (lanes 2–12) of EGF. Either buffer (lane 2), or the indicated amounts of control GST protein (lanes 2–6) or GST-SH2 domain fusion protein (lanes 7–12) were added before activation. METHODS. Fractions from unstimulated A431 cells were prepared and reactions assayed as described in Fig. 2. Control experiments (data not shown), demonstrated that neither the GST nor the GST-SH2 fusion protein inhibited SIF DNA-binding when added after SIF activation. GST and GST-GRB2 (residues 50–161) were prepared as described²³ and provided by J. Schlessinger (NYU Medical Center).

added a monoclonal antiphosphotyrosine antibody (4G10, ref. 21) to the activation reaction. The antiphosphotyrosine antibody, but not a control antibody (12CA5, ref. 22), inhibited SIF activation in a concentration-dependent manner (Fig. 4a, compare lanes 3–5 with 9–11). Inhibition was partially reversed by preincubating the antibody with free phosphotyrosine but not phosphoserine or phosphothreonine (data not shown). In contrast, when anti-phosphotyrosine antibody was added after activation but before mobility-shift assay, inhibition was not observed (lanes 6–8). Thus, the antiphosphotyrosine antibody inhibits activation of SIF rather than SIF DNA-binding activity.

Signal transduction by receptor tyrosine kinases involves interaction with proteins containing SH2 domains^{8,9}. To determine whether phosphotyrosine-SH2 domain interactions were required for SIF activation *in vitro*, we added an *Escherichia coli* produced SH2 domain as a competitive inhibitor (Fig. 4b). A glutathione-S-transferase (GST) fusion protein containing the SH2 domain from GRB2 (ref. 23) inhibited SIF activation in a dose-dependent fashion (lanes 8–12). Equal amounts of GST protein had no effect (lanes 3–7), indicating that inhibition was due to the SH2 moiety of the fusion protein. As with the anti-phosphotyrosine antibody, inhibition was observed only when the GST-SH2 protein was added before receptor activation, not when added subsequent to activation (data not shown). These data suggest that phosphotyrosine-SH2 domain interactions are required for SIF activation *in vitro*.

We have shown that activation of SIF DNA-binding activity by EGF *in vitro* proceeds by a reaction requiring receptor, ATP, and phosphotyrosine-SH2 domain interactions, although the mechanism of activation remains unknown. In the simplest model, SIF itself or a component may be recruited to the receptor and directly phosphorylated on tyrosine, as recently reported for the interferon-activated factor ISGF-3 (refs 24–27). Alternatively, there may be one or more intermediate events in SIF activation. Our data suggest that at least one phosphotyrosine-SH2 interaction is required for SIF activation. Although the GRB2 protein was the source of the SH2 domain used in our experiment, we do not regard this as evidence for a role for GRB2 in SIF activation. Other SH2 domains may inhibit SIF activation at much lower concentrations. Indeed, this assay may be useful for studying the biochemical specificity of phosphotyrosine-SH2 recognition and for the testing of potential inhibitors of this interaction. □

A new component of the transcription factor DRTF1/E2F

Rowena Girling, Janet F. Partridge*, Lasantha R. Bandara, Neil Burden, Nicholas F. Totty†, J. Justin Hsuan† & Nicholas B. La Thangue‡

Laboratory of Eukaryotic Molecular Genetics, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

† Ludwig Institute for Cancer Research, Middlesex Hospital/University College Branch, Courtauld Building, 91 Riding Street, London W1P 8BT, UK

TRANSCRIPTION factor DRTF1/E2F coordinates events in the cell cycle with transcription by its cyclical interactions with important regulators of cellular proliferation like the retinoblastoma tumour-suppressor gene product (Rb) and the Rb-related protein, p107 (refs 1–8). DRTF1/E2F binding sites occur in the control regions of genes involved in proliferation^{9,10}, and both Rb and p107 repress the capacity of DRTF1/E2F to activate transcription (refs 11, 12; M. Zamanian and N.B.L.T., manuscript submitted). Mutant Rb proteins isolated from tumour cells are unable to bind DRTF1/E2F (refs 11–13), and certain viral oncoproteins, such as adenovirus E1A, sequester Rb and p107 in order to free active DRTF1/E2F (refs 5, 11, 12, 14, 15). Here we report the isolation of a complementary DNA encoding DRTF1-polypeptide-1 (DP-1), a major sequence-specific binding protein that is present in DRTF1/E2F, including Rb- and p107-associated DRTF1/E2F. The DNA-binding domain of DP-1 contains a region that resembles that of E2F-1 (refs 16, 17), and recognizes the same sequence. DRTF1/E2F thus appears to contain at least two sequence-specific DNA-binding proteins.

E2F was defined as an activity induced in HeLa cells after adenovirus infection¹⁸, whereas DRTF1 was defined as a transcription factor downregulated during the differentiation of F9 embryonal carcinoma (EC) cells^{19,20}. As they bind to similar DNA sequences and associate with the same proteins^{5,6,8,21–24}, they are likely to be closely related. Here we refer to the DNA binding activity in whole cell extracts which recognizes the E2F site as DRTF1/E2F.

DRTF1 was purified from F9 EC whole cell extracts by sequential applications to a DNA-binding-site affinity matrix containing either a wild-type or mutant binding site²⁵. The DNA-binding site was taken from a region of the adenovirus E2A promoter (–71 to –50) that contains a high-affinity E2F binding site²⁰. We routinely purified a group of polypeptides (Fig. 1a, track 2) that could activate transcription *in vitro* in a binding site-dependent fashion²⁵. Several polypeptides in the affinity-purified material specifically bound to the wild-type site (Fig. 1a, compare tracks 5 to 6; ref. 25). One polypeptide (*M*_r ~46,000 (46K); Fig. 1a, track 2, indicated as p46) was excised and digested with a lysylendopeptidase; ten of the resulting fragments were purified and sequenced (Fig. 1b; peptides boxed). We predicted the DNA sequences that encode two of the peptides (Fig. 1b, legend) and then used these as primers to amplify a cDNA fragment derived from F9 EC RNA. Clones representing the complete coding sequence of p46 (referred to as DP-1) were finally isolated from an F9 EC cDNA library.

The complete DP-1 coding sequence contains an open reading frame encoding 429 in-frame amino acids that includes eight of the peptides from the p46 sequence (Fig. 1b). The cDNA probably includes the initiating methionine as there is an in-frame termination codon immediately upstream and because the sequence flanking and including this methionine codon would be an efficient translation initiation signal²⁶. By northern analysis

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* Present address: Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104, USA.

‡ To whom correspondence should be addressed.

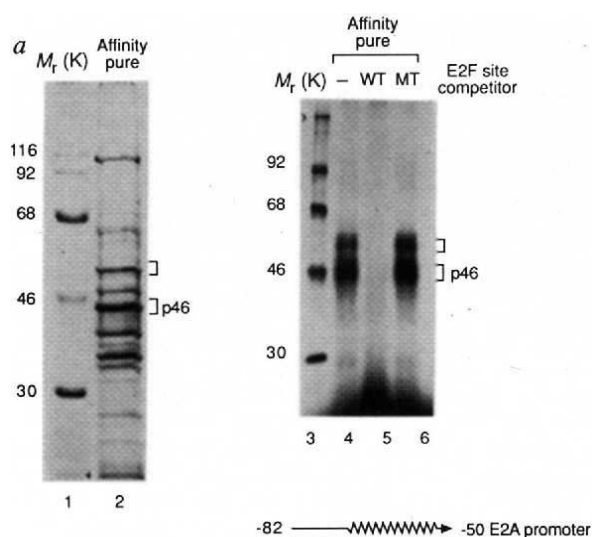
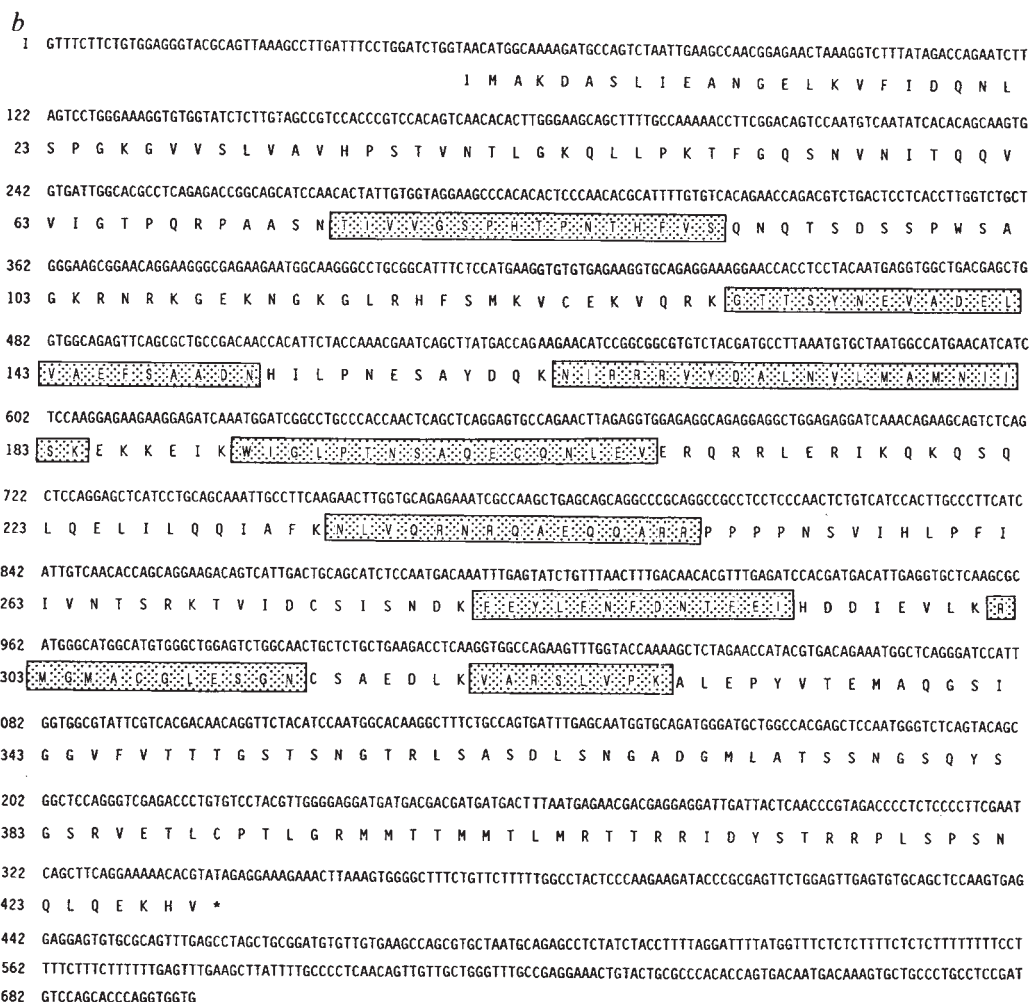


FIG. 1 *a*, Affinity purification of DRTF1/E2F from F9 EC cells. Polypeptides in affinity-purified DRTF1/E2F (about 5 μ g from about 5×10^{10} F9 EC cells) were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue (track 2); track 1 shows M_r standards. DNA-binding polypeptides in affinity-purified DRTF1/E2F were assayed by crosslinking to the adenovirus E2A promoter distal E2A site (binding site details indicated at bottom right) either in the absence (track 4) or presence of competing wild-type (track 5) or mutant (track 6) E2F binding sites; M_r standards shown in track 3. The p46 polypeptide is indicated; upper bracket shows another group of polypeptides which also specifically bind to the E2F site ($M_r \sim 55,000$). *b*, Nucleotide sequence of cDNA encoding murine DP-1 and predicted amino acid sequence. Boxed sequences are peptides obtained from microsequencing of DP-1. Two peptide sequences could not be accounted for from the cDNA sequence. *c*, Comparison of DP-1 and E2F-1. DNA-binding domains in DP-1 and E2F-1 (amino acid 84–204 in DP-1, and 89–191 in E2F-1; ref. 16). A region of significant similarity (amino acid 163–236 in DP-1, 162–226 in E2F-1) includes sequences outside the DNA-binding domain. *d*, Alignment of amino-acid sequences

of DP-1 and E2F-1 in regions of highest similarity. Heavy bars indicate identical and light bars similar amino-acid residues. *e*, Similar regions of DP-1 and E2F-1 form an amphipathic α -helix. DP-1 (amino acids 167–183) and E2F-1 (amino acids 166–182) regions of similarity are shown as a helical wheel. Amino acids are represented in one-letter notation. **METHODS.** DRTF1/E2F was affinity-purified from extracts from whole F9 EC cells²⁵ using affinity matrices containing the wild-type E2F site from the adenovirus E2A promoter (–71 to –50); the binding activity was also applied to a matrix containing an E2F binding site mutated in nucleotides –62 to –60. About 5.0 μ g protein was purified from $\sim 10^{10}$ F9 EC cells. For UV

of poly(A)⁺ F9 EC RNA, the DP-1 transcript was estimated as ~ 2.8 kilobases (kb) (data not shown); it is expressed in many different cell types and in a wide range of tissues during murine embryogenesis (J.F.P., P. Tassios and N.B.L.T., manuscript in preparation). Homology searches of the Leeds and Swiss protein data bases failed to detect any significant similarity with any other protein. But a small region within the DNA-binding domain of DP-1 has significant similarity to an analogous region in the DNA-binding domain of E2F-1.

To confirm that DP-1 is a component of DRTF1/E2F, peptide antisera were raised against DP-1 (Fig. 2*e*). Immunoblotting affinity-purified DRTF1/E2F with anti-peptide 15 revealed two polypeptides with apparent M_r s 46K and 55K (Fig. 2*a*, track 2) that were recognized by the antiserum (Fig. 2*a*; compare tracks 5 and 6). We confirmed that DP-1 was part of the DRTF1/E2F DNA-binding complex in F9 EC cell extracts from which DRTF1 resolves as complexes DRTF1*a*, *b* and *c* (ref. 25). Addition of anti-peptide A, but not the preimmune serum, causes either a supershift and concomitant reduction in DRTF1*a*, *b* and *c* (Fig. 2*b*, antiserum 3; compare tracks 1 and 2) or abolishes all DRTF1 complexes (Fig. 2*b*, antiserum 4; compare tracks 5 and 6). The effects of both antisera 3 and 4 could be competed



crosslinking of DRTF1/E2F to the E2F site²⁵, we used ~ 50 ng of affinity-purified protein. A 100-fold molar excess of either wild-type (–71 to –50) or mutant oligonucleotides was used in competition assays. Affinity-purified DRTF1 was precipitated with TCA, electrophoresed through SDS-polyacrylamide, and p46 electroeluted; p46 was confirmed as pure by electrophoresis and silver staining of eluted material. p46 was digested with a lysylendopeptidase, and peptides purified by HPLC using Aquapore AX-300 and RP-300 in series in 0.1% TFA with a 1% per min acetonitrile gradient. Peak fractions were sequenced by automated Edman degradation in a 477A/120A gas-liquid pulse sequencer (Applied Biosystems). A set of degenerate oligo-

by peptide A but not the unrelated peptide 1 (Fig. 2b; compare tracks 3, 4 and 7, 8). Extracts from other cell types, including HeLa, 293, PCC3, PCC4, SAOS-2, and several SV40-transformed mouse-cell lines gave similar results (data not shown). We conclude that DP-1 is a component of DRTF1/E2F and that it is present in all DRTF1/E2F DNA-binding complexes in F9 EC cell extracts.

When anti-peptide A was incubated with affinity-purified DRTF1/E2F, a supershift was apparent (Fig. 2c; compare tracks 1, 2 and 5, 6; clearest for antiserum 3). The effect was not as marked as in F9 EC cell extracts, probably because some of the DP-1 polypeptide loses its N-terminal region during purification (data not shown). Therefore we used anti-peptide 18 (Fig. 2e; antiserum 11), representing sequence from the DNA-binding domain of DP-1, which specifically inhibits the DNA-binding activity of affinity-purified DRTF1/E2F (Fig. 2c; compare tracks 11, 12).

We assessed whether DP-1 was present in DRTF1/E2F complexes that form on different E2F binding sites using the class 1 and class 2 consensus E2F binding sites which were defined by binding site selection²⁷ and occur in the transcriptional control regions of certain cellular genes^{9,10,28}. Anti-peptide A abolishes DRTF1/E2F complexes formed on both class 1 and class 2 binding sites (Fig. 2d; compare tracks 3, 4 and 7, 8).

To determine whether DP-1 is present in complexes containing Rb, we immunoprecipitated them with an anti-Rb mono-

clonal antibody²⁹ from extracts of the human leukaemic cell line JM, which contain high levels of the DRTF1/E2F-Rb complex⁵. DRTF1/E2F DNA-binding activity was released from Rb by detergent, but not from the immunoprecipitate with the control monoclonal antibody A7 (Fig. 3a; compare tracks 2 and 3, and respective depleted extracts, tracks 4 and 5). The presence of DP-1 in Rb-associated DRTF1/E2F was tested with anti-peptide A (antiserum 3) and anti-peptide 18

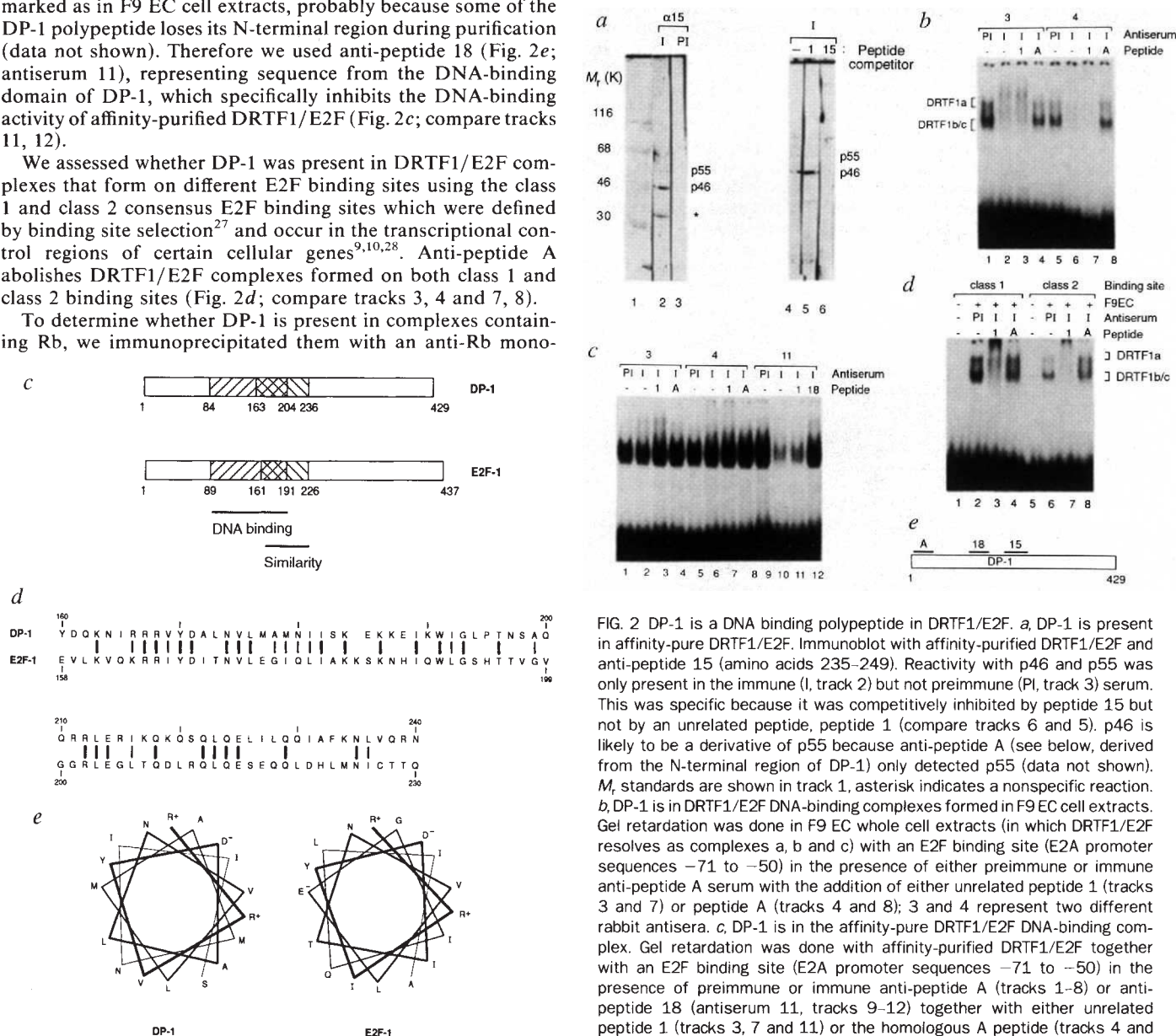


FIG. 2 DP-1 is a DNA binding polypeptide in DRTF1/E2F. **a**, DP-1 is present in affinity-pure DRTF1/E2F. Immunoblot with affinity-purified DRTF1/E2F and anti-peptide 15 (amino acids 235–249). Reactivity with p46 and p55 was only present in the immune (I, track 2) but not preimmune (PI, track 3) serum. This was specific because it was competitively inhibited by peptide 15 but not by an unrelated peptide, peptide 1 (compare tracks 6 and 5). p46 is likely to be a derivative of p55 because anti-peptide A (see below, derived from the N-terminal region of DP-1) only detected p55 (data not shown). **b**, DP-1 is in DRTF1/E2F DNA-binding complexes formed in F9 EC cell extracts. Gel retardation was done in F9 EC whole cell extracts (in which DRTF1/E2F resolves as complexes a, b and c) with an E2F binding site (E2A promoter sequences –71 to –50) in the presence of either preimmune or immune anti-peptide A serum with the addition of either unrelated peptide 1 (tracks 3 and 7) or peptide A (tracks 4 and 8); 3 and 4 represent two different rabbit antisera. **c**, DP-1 is in the affinity-pure DRTF1/E2F DNA-binding complex. Gel retardation was done with affinity-purified DRTF1/E2F together with an E2F binding site (E2A promoter sequences –71 to –50) in the presence of preimmune or immune anti-peptide A (tracks 1–8) or anti-peptide 18 (antiserum 11, tracks 9–12) together with either unrelated peptide 1 (tracks 3, 7 and 11) or the homologous A peptide (tracks 4 and 8) or 18 (track 12). **d**, DP-1 is in DRTF1/E2F DNA binding complexes that form on divergent E2F binding sites. Gel retardation was done in F9 EC whole cell extracts with either a class 1 (tracks 1–4) or class 2 (tracks 5–8) binding site in the presence of either preimmune or immune anti-peptide A (antiserum 4), together with either unrelated peptide 1 (tracks 3 and 7) or peptide A (tracks 4 and 8). **e**, Location of peptides A, 18 and 15 in DP-1. **METHODS**. Peptides 15, A or 18 were coupled to keyhole limpet haemocyanin and used to immunize rabbits. Generation of antibodies and immunoblotting followed standard procedures. In gel retardation assays ~5.0 ng affinity-purified DRTF1/E2F or ~5.0 µg of F9 EC crude cell extract were assayed with an oligonucleotide containing E2A promoter sequences –71 to –50 in the presence of ~100-fold molar excess of –62/–60 (E2A sequences –71 to –50 mutated in positions –62, –61 and –60 (ref. 20)). In **d**, class 1 (TTTGGCGGGAAT) or class 2 (ATTTGGCGGGAAT) binding sites²⁷ were used. Antisera were added during the preincubation period.

nucleotide primers were synthesized on the basis of amino-acid sequence in peptides 6 (75–91) and 5 (235–249) as follows: C-terminal regions of peptide 6 (PNTHFV), 5'-CGCGGATCCCC(ACGT)AA(CT)AC(ACGT)CA(CT)-TT(CT)GT-3'; and peptide 5 (AQESQN), antisense strand 5'-CGCGGATCCA-(AG)(AG)TT(CT)TG(ACGT)(CG)(AT)(CT)TC(CT)TG(ACGT)GC-3'; both oligonucleotides included a linker sequence at the 5' end. Peptide 5 antisense oligonucleotide was used to synthesize cDNA from F9 EC cell RNA which was then used in a PCR with both peptide 6 and 5 primers. Products were subcloned, sequenced and cDNAs derived from DP-1 RNA identified by the presence of peptides 21 and 3, two further peptides obtained by micro-sequencing peptides derived from p46, which are located between peptides 6 and 5. This cDNA fragment was used to screen several murine cDNA libraries. DP-1 cDNA clones were frequently rearranged and the nucleotide sequence shown is from cDNAs from an F9 EC cell library.

FIG. 3 DP-1 in pRb and p107-DRTF1/E2F complexes. *a*, DP-1 associates with Rb. Immunoprecipitation was from JM cell extracts with either anti-Rb monoclonal antibody IF8 (ref. 29) or a control monoclonal antibody A7. Immunoprecipitates were treated with 1.0% deoxycholate and the released material assayed for DRTF1/E2F activity in gel retardation. DRTF1/E2F binding activity was only detected in the anti-Rb immunoprecipitates (compare tracks 2 to 3); the JM cell extract depleted with anti-Rb had reduced levels of the Rb complex (compare tracks 4 to 5, depleted complex indicated by 'a'). Detergent-released DRTF1/E2F was further assayed for reactivity with anti-peptide A (antiserum 3; tracks 6 and 7) and anti-peptide 18 (antiserum 11; tracks 8 and 9) in the presence of either unrelated peptide 1 (tracks 6 and 8) or the homologous peptides (tracks 7 and 9). Anti-peptide A caused a supershift (indicated by 's' in track 6), whereas anti-peptide 18 interfered with DRTF1/E2F DNA-binding activity (compare tracks 8 and 9). *b*, DP-1 associates with p107; DRTF1/E2F was resolved in an F9 EC cell extract in the presence of either the control monoclonal antibody (A7; track 1), anti Rb (IF8; track 2), or anti-p107 (SD9; track 3); note that anti-p107 reduced level of DRTF1a. In tracks 4 and 5, JM cell extracts were immunoprecipitated with anti-p107 monoclonal antibody. DRTF1/E2F was released as described in *a*, and assayed for reactivity with anti-peptide 18 (antiserum 11) in the presence of either unrelated peptide 1 (track 4) or the homologous peptide 18 (tracks 5). Note that anti-peptide 18 caused a reduction in DRTF1/E2F DNA-binding activity (compare tracks 4 and 5).

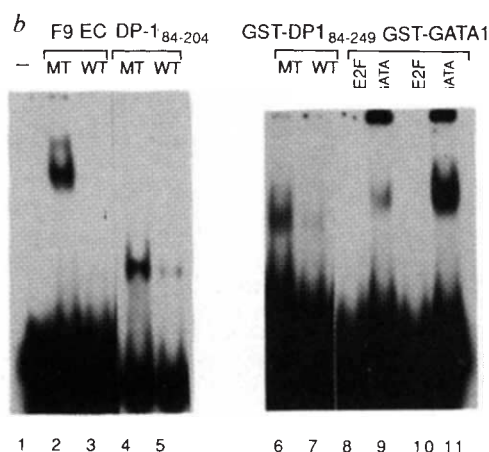
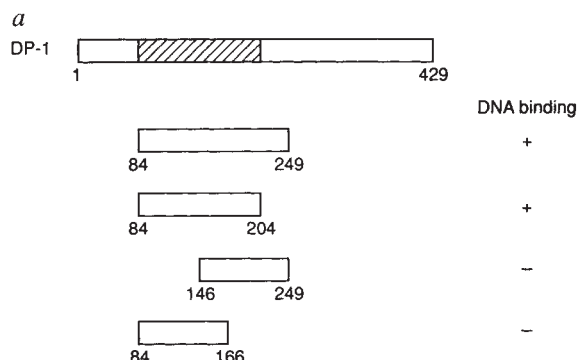
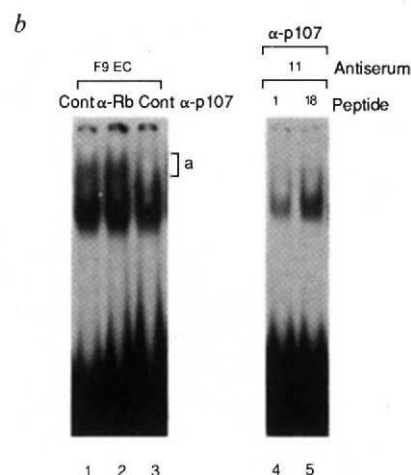
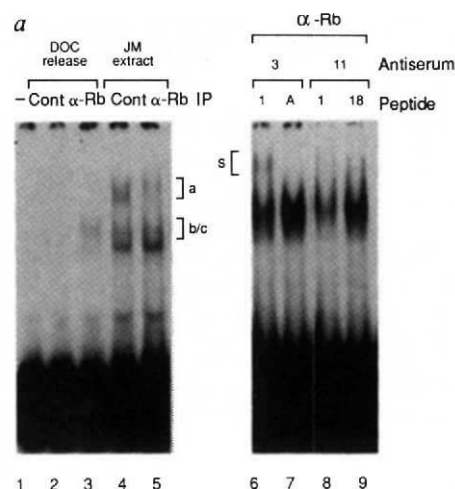


FIG. 4 DP-1 is a sequence-specific DNA binding protein. *a*, Summary of DNA binding properties of DP-1 fusion proteins. The indicated regions of DP-1 were expressed as fusion proteins, affinity-purified and assayed for DNA-binding activity in a gel retardation assay. *b*, DP-1₈₄₋₂₀₄ is sufficient for sequence-specific DNA binding to an E2F site. Affinity-purified GST-DP-1₈₄₋₂₀₄ was cleaved with thrombin and the released polypeptide assayed (~1.0 µg) for binding to the E2F site in the presence of ~100-fold excess of competing mutant (track 4) or wild-type (track 5) E2F site (the distal E2F site, -71 to -50, from the adenovirus E2A promoter). Competition with the same binding sites in F9 EC cell extracts is shown in tracks 2 and 3; tracks



1 to 5 were taken from the same experiment. GST-DP-1₈₄₋₂₄₉ had similar DNA specificity (compare tracks 6 and 7; about 2.5 µg of uncleaved fusion protein in the presence of 100-fold excess of competing mutant or wild-type binding site), whereas DP-1₁₄₆₋₂₄₉ and DP-1₈₄₋₁₄₆ failed to bind in these conditions (data not shown). Likewise, GST-GATA 1 (~1.5 µg) failed to bind to the E2F site (tracks 8 and 10) but did to its cognate binding site (tracks 9 and 11) in the presence (tracks 8 and 9) or absence (tracks 10 and 11) of nonspecific salmon sperm DNA (2 µg); tracks 6 to 11 are from the same experiment.

METHODS. Regions of the DP-1 cDNA were amplified in a PCR and subcloned into pGEX-2T (ref. 13). Oligonucleotide primers used in each PCR were as follows: DP-1₈₄₋₂₄₉, 5'-CGCGGATCCCCAACACGCATTTTGTG-3' and 5'-CGCGGATCCCCCTGCGGGCCTGCTG-3'; DP-1₈₄₋₂₀₄, 5'-CGCGGATCCCCAACACGCATTTTGTG and CGCGGATCCGTCTCTGGCACTCTGAGC; DP-1₁₄₆₋₂₄₉, 5'-CGCGGATCCTTCAGCGCTGCCGACAACCAC and 5'-CGCGGATCCCCCTGCGGGCCTGCTG; DP-1₈₄₋₁₆₆, 5'-CGCGGATCCCCAACACGCATTTTGTG and CGCGGATCCCCGGATGTTCTTCTGGTC. GST fusion proteins were affinity-purified on glutathione-sepharose and their purity assessed by PAGE (ref. 13). Gel retardation with the distal E2F binding site from the adenovirus E2A promoter (-71 to -50 region) and the DP-1 derivatives was done in the absence of salmon sperm DNA (tracks 4, 5, 6 and 7). Thrombin digestion of GST-DP-1₈₄₋₂₀₄ was by resuspending the GST-fusion protein/glutathione-sepharose beads in 50 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM CaCl₂, 0.1% β-mercaptoethanol containing human thrombin at 3 µg ml⁻¹ for 2 h. The presence of cleaved DP-1₈₄₋₂₀₄ in the supernatant was confirmed by immunoblotting. GST-GATA-1 contained a full-length murine GATA-1 coding sequence³⁰ and was incubated with a synthetic GATA-1 binding site.

(antiserum 11). Anti-peptide A caused a supershift (Fig. 3a, compare tracks 6 and 7; shift indicated by 's'), whereas anti-peptide 18 caused a reduction in DRTF1/E2F DNA-binding activity (Fig. 3a; compare tracks 8 and 9). We conclude that DP-1 co-immunoprecipitates with Rb from JM cell extracts and is therefore likely to associate with Rb *in vivo*.

In F9 EC cell extracts, an anti-p107 antibody disrupted DRTF1a (Fig. 3b; compare tracks 2 and 3), which, together with our result that the anti-DP-1 peptide antibodies interfered with DRTF1a (Fig. 2b), suggested that p107 and DP-1 exist in the same DRTF1/E2F complex. Furthermore, DRTF1/E2F DNA binding activity released from an anti-p107 immunoprecipitation was specifically reduced by anti-peptide 18 (Fig. 3b; compare track 4 and 5). We conclude therefore that DP-1 can exist in DRTF1/E2F complexes that contain either Rb or p107.

To determine whether DP-1 could bind to the E2F site in a sequence-specific way, regions were expressed as glutathione-S-transferase (GST) fusion proteins (Fig. 4a), affinity-purified and tested for DNA-binding activity. The smallest region defined that retains sequence-specific DNA-binding activity contains DP-1 protein sequence from amino-acid residue 84 to 204 (DP-1₈₄₋₂₀₄; Fig. 4b, compare tracks 4 and 5); in the same conditions, an unrelated sequence-specific transcription factor, GATA-1 (ref. 30), failed to bind to the wild-type E2F site (Fig. 4b; compare tracks 8, 9 and 10, 11). Smaller regions of DP-1 (DP-1₁₄₆₋₂₄₉ and DP-1₈₄₋₁₆₆) failed to bind to the E2F site, whereas DP-1₈₄₋₂₄₉ retained sequence-specific DNA binding (Fig. 4a and b; compare track 6 and 7). Thus DP-1 has DNA-binding specificity which is similar to DRTF1/E2F.

We noticed a region (DP-1 residues 160–200) with significant similarity to a sequence in the DNA-binding domain of E2F-1 (Fig. 1c), in which 42% is identical and 70% similar (Fig. 1d). These regions could include two α -helices, one of which would be amphipathic (helical wheel in Fig. 1e). As DP-1 and E2F-1 bind to the same sequence (this work, and refs 16, 17), this region of similarity may be involved in recognizing an E2F binding site. This idea is supported by the potential similarity of two other DNA-binding proteins that regulate transcription during the yeast cell cycle. These proteins, encoded by *SW14* and *cdc10*, bind to a DNA sequence that resembles the E2F site³¹, and contain a region within their DNA-binding domains with features in common with the DP-1/E2F-1 α -helical region already discussed (Fig. 1e, and ref. 32).

Another region of similarity between DP-1 and E2F-1 is apparent outside the DNA-binding domain (DP-1 amino acids 210–240, 41% identity; Fig. 1d) which, like the earlier region, could form an amphipathic α -helix. It is possible that DP-1 and E2F-1 interact because DP-1 is present in all DRTF1/E2F complexes, some of which contain E2F-1, rather than in the transcription factor AP-1, in which different proteins interact through related domains³³.

The region of similarity between DP-1 and E2F-1 is likely to be an important part of their DNA-binding domains, but we cannot distinguish whether it is involved in recognizing the E2F sequence *per se* or if it provides a dimerization domain which then allows sequence-specific recognition. We believe, however, that it includes the DNA-recognition domain because of its distant, but significant, similarity with the DNA-binding domains of the yeast cell cycle-regulating proteins *SW14* and *cdc10* (ref. 32). □

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Recognition of a high-affinity phosphotyrosyl peptide by the Src homology-2 domain of p56^{lck}

Michael J. Eck[†]*, Steven E. Shoelson[‡] & Stephen C. Harrison[§]*

* Howard Hughes Medical Institute and Laboratory of Molecular Medicine, Children's Hospital, 300 Longwood Avenue, Boston, Massachusetts 02115, USA

† Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA

‡ Research Division, Joslin Diabetes Center, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA

§ Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138, USA

THE Src homology-2 (SH2) domains are modules of about 100 amino-acid residues that are found in many intracellular signal-transduction proteins^{1,2}. They bind phosphotyrosine-containing sequences with high affinity and specificity^{1–4}, recognizing phosphotyrosine in the context of the immediately adjacent polypeptide sequence^{3–12}. The protein p56^{lck} (Lck) is a Src-like, lymphocyte-specific tyrosine kinase^{13–15}. A phosphopeptide library screen has recently been used to deduce an 'optimal' binding sequence for the Lck SH2 domain¹⁶. There is selectivity for the residues Glu, Glu and Ile in the three positions C-terminal to the phosphotyrosine. An 11-residue phosphopeptide derived from the hamster polyoma middle-T antigen, EPQpYEEIPIYL, binds with an approximately 1 nM dissociation constant to the Lck SH2 (ref. 17), an affinity equivalent to that of the tightest known SH2-phosphopeptide complex. We report here the high-resolution crystallographic analysis of the Lck SH2 domain in complex with this phosphopeptide. Recent crystallographically derived structures of the Src SH2 domain in complex with low-affinity peptides¹⁸, which do not contain the EEI consensus, and NMR-derived structures of unliganded Abl (ref. 19) and p85 (ref. 20) SH2 domains have revealed the conserved fold of the SH2 domain and the properties of a phosphotyrosine binding pocket. Our high-affinity complex shows the presence of a second pocket for the residue (pY+3) three positions C-terminal to the phosphotyrosine (pY). The

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peptide is anchored by insertion of the pY and pY+3 side chains into their pockets and by a network of hydrogen bonds to the peptide main chain. In the low-affinity phosphopeptide/Src complexes¹⁸, the pY+3 residues do not insert into the homologous binding pocket and the peptide main chain remains displaced from the surface of the domain.

The conserved folded structure of an SH2 domain contains a central β -sheet, flanked on either face by two α -helices. To facilitate comparison of SH2 domains, for which over 60 sequences have already been published^{1,21}, we propose a notation for

secondary-structure elements (Figs 1 and 2). The strands are called βA – βG ; the helices, αA and αB . Loops are named by the letters of the secondary structures they join, and residues are numbered sequentially within each structural element. We number phosphopeptide residues from the phosphotyrosine as pY+1, pY+2, and so on. The central sheet of the SH2 domain (Fig. 1a) can be described as a three-strand antiparallel ' β -meander', augmented by parallel strands, βA and βG , at one edge. Helix αA packs across the 'proximal' or 'phosphotyrosine' face of the central sheet; βD diverges towards the surface

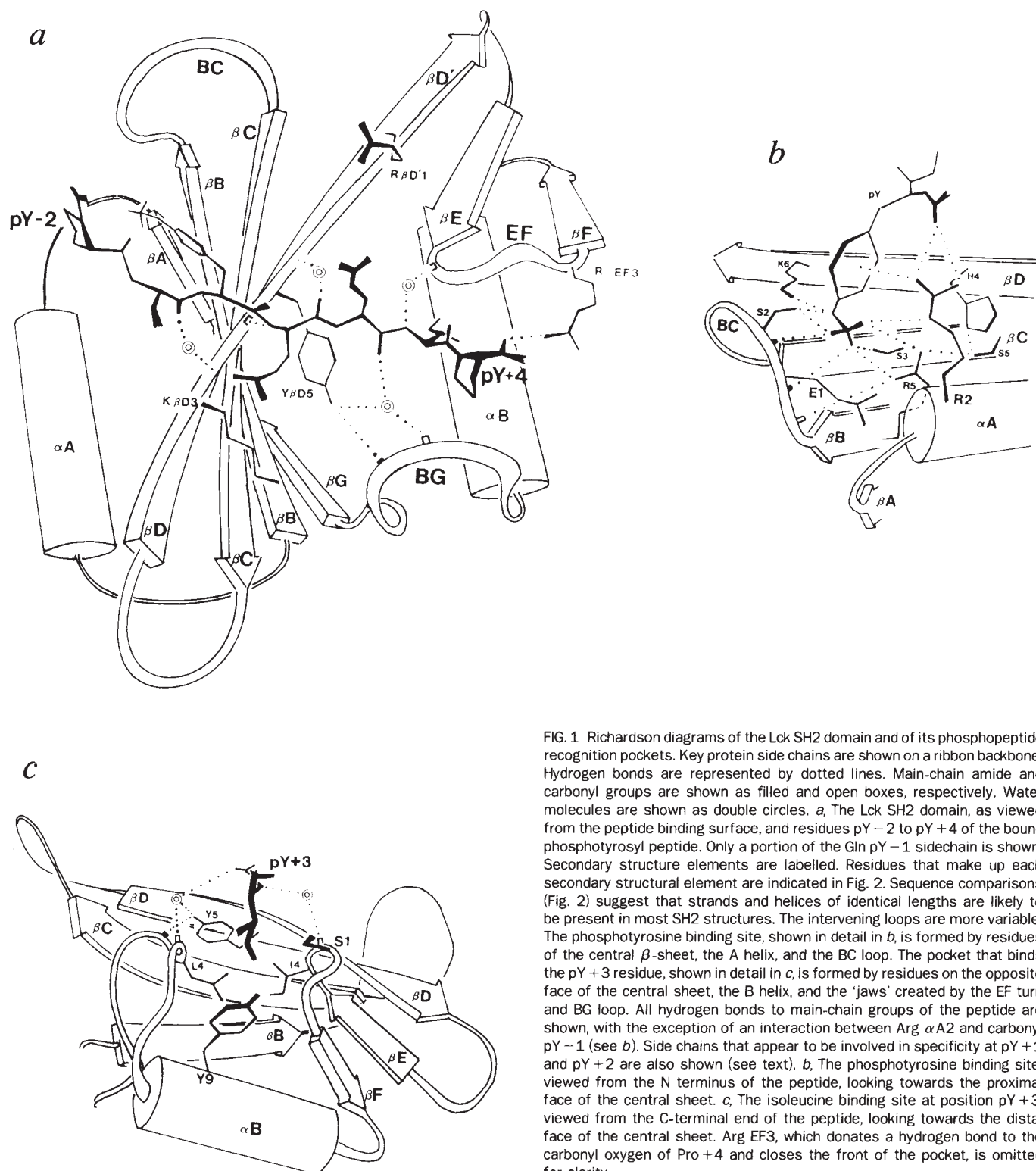


FIG. 1 Richardson diagrams of the Lck SH2 domain and of its phosphopeptide recognition pockets. Key protein side chains are shown on a ribbon backbone. Hydrogen bonds are represented by dotted lines. Main-chain amide and carbonyl groups are shown as filled and open boxes, respectively. Water molecules are shown as double circles. *a*, The Lck SH2 domain, as viewed from the peptide binding surface, and residues pY-2 to pY+4 of the bound phosphotyrosyl peptide. Only a portion of the Gln pY-1 sidechain is shown. Secondary structure elements are labelled. Residues that make up each secondary structural element are indicated in Fig. 2. Sequence comparisons (Fig. 2) suggest that strands and helices of identical lengths are likely to be present in most SH2 structures. The intervening loops are more variable. The phosphotyrosine binding site, shown in detail in *b*, is formed by residues of the central β -sheet, the A helix, and the BC loop. The pocket that binds the pY+3 residue, shown in detail in *c*, is formed by residues on the opposite face of the central sheet, the B helix, and the 'jaws' created by the EF turn and BG loop. All hydrogen bonds to main-chain groups of the peptide are shown, with the exception of an interaction between Arg $\alpha A2$ and carbonyl pY-1 (see *b*). Side chains that appear to be involved in specificity at pY+1 and pY+2 are also shown (see text). *b*, The phosphotyrosine binding site, viewed from the N terminus of the peptide, looking towards the proximal face of the central sheet. *c*, The isoleucine binding site at position pY+3, viewed from the C-terminal end of the peptide, looking towards the distal face of the central sheet. Arg EF3, which donates a hydrogen bond to the carbonyl oxygen of Pro +4 and closes the front of the pocket, is omitted for clarity.

opposite αA (the 'distal' face). It continues as $\beta D'$ and forms a second, smaller sheet with strands E and F (Fig. 1a). This second sheet is joined by helix αB to a prominent loop (BG), which doubles back and connects into the central sheet. The BG loop and the EF turn face each other from opposite ends of αB , creating a set of 'jaws', which partly define the specificity pocket for peptide residue pY+3 (see later).

The 11-residue peptide binds in an extended conformation across the D-strand edge of the central sheet (Fig. 1a). Seven of the residues, from pY-2 to pY+4, make some contact with the protein, and all are well defined in the electron density map (Fig. 3). The N-terminal and the three C-terminal residues of the peptide turn away from the protein surface, and they lie in relatively weaker electron density. The four residues from pY to pY+3 are particularly tightly anchored. The rather flat surface formed by residues $\beta D4$ - $\beta D6$ supports pY+1 and pY+2, whereas pY and pY+3 'drop' over the edge of this surface into deep specificity pockets on the proximal and distal sides respectively (Fig. 4). At the proximal end, the peptide conformation around the phosphotyrosine is fixed by hydrogen bonds between Arg $\alpha A2$ and the main-chain carbonyl of pY-1 and between the carbonyl of $\beta D4$ and the main-chain amide of pY+1. At the distal end, a hydrogen bond from the side chain of Arg EF3 anchors the carbonyl of pY+4. Four bound waters provide additional main-chain constraints (Fig. 1a). One of these waters bridges between the carbonyl of pY-1 and the amide of residue $\beta D4$; a second, between the carbonyl of pY+1 and the amide of $\beta D6$. The remaining two waters clamp the pY+2/pY+3 peptide bond.

The interactions in the phosphotyrosine pocket (Fig. 1b) are very similar to those described for the Src complex¹⁸. Its side chain lies in a deep, elongated groove formed by parts of the B, C and D strands, the A helix, and the BC ('phosphate-binding') loop. The side chains of His $\beta D4$ and Lys $\beta D6$ and the main-chain atoms of Tyr $\beta D5$ form the semicircular rim of the binding groove and cradle the aromatic ring of the phosphotyrosine. The phosphate forms the centre of an elaborate hydrogen-bonding network (Fig. 1b). Two arginine residues are of particular importance. Arginine $\alpha A2$ donates four hydrogen bonds to the peptide: one to a phosphate oxygen, one amino-aromatic bond from N η 1 to the phosphotyrosine ring, and two to the main-chain carbonyl of pY-1. It is therefore critical for the conformation of the entire phosphotyrosine residue. Arg

$\beta B5$ makes bidentate hydrogen bonds with the phosphate group. Other hydrogen bonds are shown in Fig. 1b. The phosphotyrosine itself is obviously critical for the hydrogen-bond network, and the entire binding groove may be destabilized in its absence. Indeed, NMR structures of the free SH2 domains from Abl and p85 show that the BC loop is poorly ordered^{19,20}. The Arg $\alpha A2$ side chain and the BC loop close in over the phosphotyrosine, and one or both must flex in order to bind or release the peptide.

The largely hydrophobic pY+3 pocket is bounded by the EF turn and the BG loop (Fig. 1c). The peptide planes between $\beta E4$ and EF1 and between BG3 and BG4 are parallel to each other, creating steeply vertical sides for the pocket. The C β of Ser EF1 also contributes. At the rear are the side chains of Ile $\beta E4$ and Leu BG4, in contact with each other, and Tyr $\beta D5$. At the bottom of the site is Tyr $\alpha B9$. The pocket is partially closed off by an arginine side chain (residue EF3), which lies across the open end and hydrogen bonds to the pY+4 peptide carbonyl. There are also two well-ordered water molecules linking the phenolic oxygen of Tyr $\alpha B9$ with the EF and BG loops, respectively. The interactions stabilizing the pY+3 pocket are largely independent of the peptide, except for the positioning of the Arg EF3 side chain. We therefore believe that the jaws are likely to be in place, even in the unliganded state. It is possible that in some SH2 domains, these loops could shift in the absence of peptide. For example, the NMR structure of the unoccupied domain from p85 suggests a poorly ordered BG loop²⁰, which probably closes when peptide binds²².

The pY+1 and pY+2 residues, both glutamic acid in this peptide, do not fit into grooves or pockets (Fig. 1a). The side chain of Glu pY+1 lies across the face of Tyr $\beta D5$; its carboxyl group is close to the amino group of Lys $\beta D3$, but the spacing is too large for a hydrogen bond. The side chain of Glu pY+2 projects away from the protein. In the crystal, it interacts with a neighbouring molecule; in solution, it could turn instead toward the side chain of Arg $\beta D'1$.

What sorts of recognition properties are suggested by the structure of this complex? The extended peptide/protein interface (500 Å²) contains 14 hydrogen bonds. Its chemical characteristics are therefore similar to those of other high-affinity protein/protein interactions^{23,24}. The contacts to peptide backbone are critical for specificity, because they impose a free-energy cost on any rearrangement needed to accommodate a

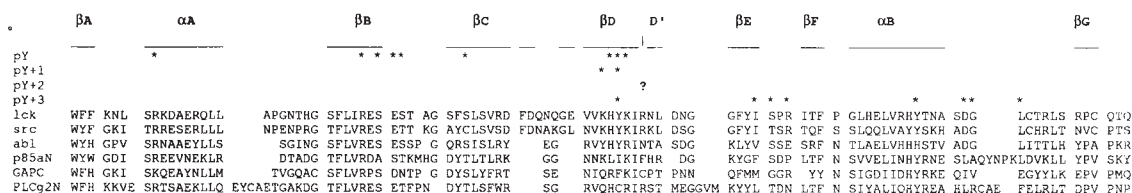


FIG. 2 Sequence alignment of several SH2 domains from proteins involved in signal transduction¹. The criteria for alignment include, in addition to available structural data: the conserved residues noted previously (Trp $\beta A1$, Arg $\alpha A2$, the 'FLVR' sequence beginning at $\beta B2$ (ref. 30), Leu $\beta C4$, Phe $\beta F3$ and Leu $\alpha B5$); the obvious pattern of sequence similarity in βC , βD and αB ; the generally hydrophobic character of $\beta E2$ -4; and the leucine two residues before the beginning of βG , as well as a proline at $\beta G2$. The last two conserved residues are important for assignment of the BG loop. The leucine (BG8 in Lck) packs against other conserved hydrophobic residues at the 'base' of the domain ($\beta B2$, $\beta C4$, $\beta C6$); the $\beta G2$ proline (conserved in most sequences, but not in Src) helps turn the G strand away from the sheet, to make room for the 'entry' of the A strand. Secondary structure assignments are indicated at the top of the figure. Hydrogen-bonding patterns have been used to decide which residues should be included in an

α -helix or a β -strand, and residues are numbered sequentially within each secondary structure element. For example, the important arginine near the N terminus of the A helix is Arg $\alpha A2$. This notation follows the convention established for the globins and other families of proteins. The helix assignments include the 'N-cap' residues; thus they differ by one residue from earlier descriptions¹⁸⁻²⁰. Two residues at the beginning of the CD loop and two residues at the end actually continue the βC : βD hydrogen-bond pattern in Lck. These are shown by short lines at the top of the figure. We have not included them in the numbering for strands C and D, because they are present only in the Src family¹. Asterisks in rows two through five indicate residues that form recognition sites for the pY to pY+3 residues respectively. Sequence alignment implies conservation of secondary structure and of most of the pY binding residues throughout all SH2 domains, and conservation of residues of the pY+3 site within the Src family.

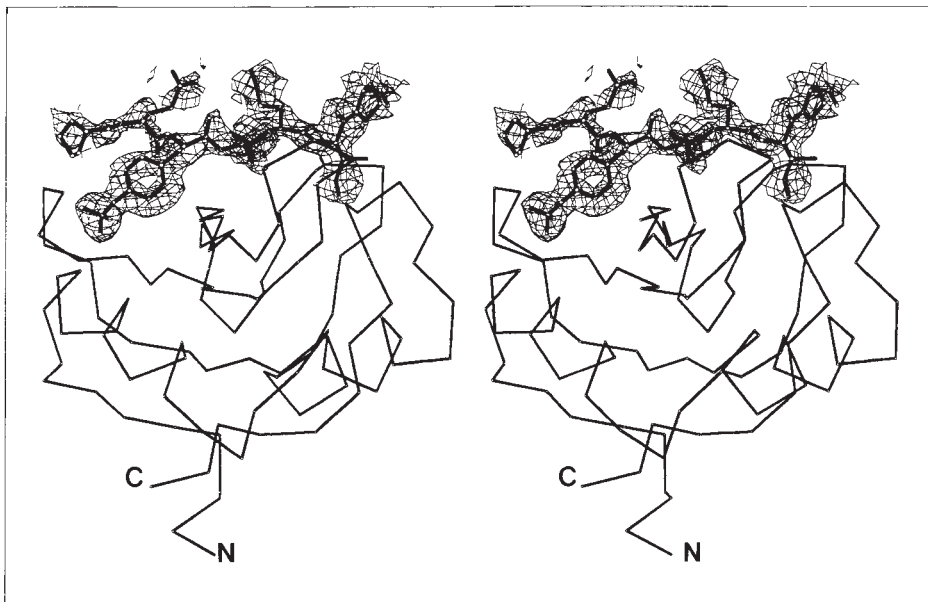
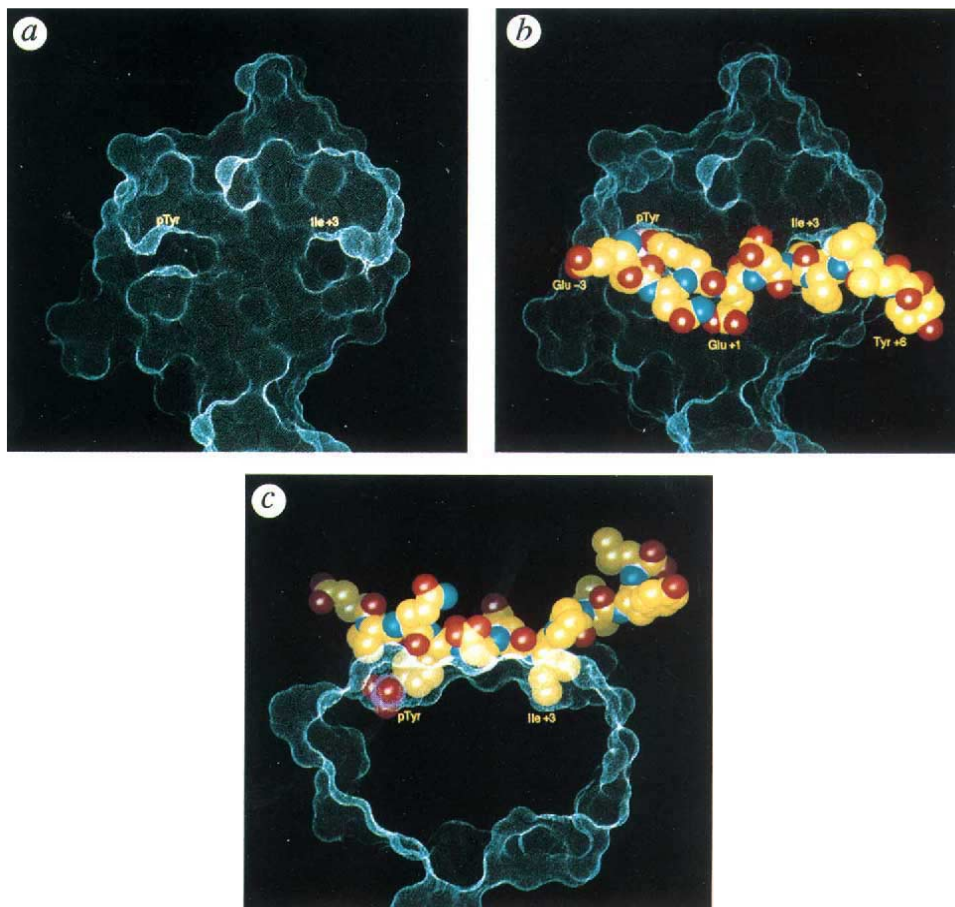


FIG. 3 Stereo diagram of the phosphopeptide electron density calculated before inclusion of peptide in the model. The map was calculated with coefficients $2|F_o| - |F_c|$, using data to 2.2 Å resolution, and it is contoured at 0.7σ above its mean value. For clarity, only contours within 2.2 Å of the peptide are plotted. The refined atomic model is displayed for peptide residues from pY-2 to pY+4. An α -carbon trace of the complete SH2 domain reveals the orientation of the bound peptide with respect to the domain. The amino- and carboxy-terminal residues are labelled. The map and model were obtained as follows. Expression and purification: A fragment of the coding region of p56^{lck} (ref. 14) between residues 119 and 226 was subcloned into the expression vector pRSET (Invitrogen). The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3)pLysS (ref. 31). Cultures were grown in LB medium containing $100 \mu\text{g ml}^{-1}$ of both ampicillin and chloramphenicol at 37 °C, induced at mid-log phase with the addition of 0.84 M IPTG and harvested 4 h later by centrifugation. Bacterial pellets were resuspended in a lysis buffer containing 50 mM Tris, pH 7.5, 200 mM NaCl, 2 mM DTT, and 1 mM PMSF. The suspension was lysed by

FIG. 4 The solvent-accessible surface of the domain reveals two deep recognition pockets. *a*, 'Top' view of the peptide binding surface. The pY binding groove and the pY+3 binding pocket are labelled. The domain is oriented as in Fig. 1*a*. *b*, A space-filling model of the 11-residue phosphopeptide has been added to the surface in *a*. The peptide binds in an extended conformation, anchored by pY and by Ile pY+3, which are 'plugged' into their respective binding pockets. Seven of the eleven peptide residues (pY-2 to pY+4) contact the surface of the protein. The surface reveals no clefts or pockets in the region of the glutamic acid residues in positions pY+1 and pY+2. These residues extend on either side of the main chain, across the relatively flat 'plateau' created by the edge of the central sheet (strand D). *c*, As in *b*, but viewed from the side. Note the surface complementarity, which extends from Pro-2 to Pro+4. N- and C-terminal to these residues, the peptide turns up and away from the surface of the protein. Solvent-accessible surfaces³⁹ were calculated using InsightII (Biosym Technologies).



non-consensus side chain. The way they position both ends of the contacting segment of the peptide suggests that tight binding will generally require the peptide conformation seen in this structure. Other high-affinity binding modes do not seem likely. In the complexes of the v-Src SH2 domain with non-specific phosphotyrosyl peptides¹⁸, the pY+3 residues are not inserted

into the homologous binding pocket, and peptide main-chain interactions are absent beyond pY+1.

What can we infer about recognition by other SH2 domains? The pY+3 pocket we have discovered explains a number of observations concerning SH2 specificity. For example, the phosphatidylinositol-3-OH kinase p85 SH2 domain binds tightly to

sonication and cleared by centrifugation for 6 h at 40,000g. The SH2 protein was contained in the supernatant and was purified by affinity chromatography on a phosphotyrosine-Sepharose column prepared by reacting two bed volumes of 20 mM *o*-phospho-L-tyrosine with CNBr-activated Sepharose beads in NaHCO₃ buffer at pH 8.3. Bound SH2 domain was eluted with the addition of 20 mM free phosphotyrosine to the wash buffer (200 mM NaCl, 2 mM DTT, 50 mM Tris, pH 7.4); gel filtration on a Superose-12 FPLC column in storage buffer (200 mM NaCl, 2 mM DTT, 20 mM BES, pH 7.0) removed phosphotyrosine and a high-molecular-weight contaminant from the affinity eluent. Crystallization and data collection: For crystallization, SH2 protein in storage buffer was concentrated to 50 mg ml⁻¹ in a Centricon-10 concentration device (Amicon) and combined with an equimolar amount of the phosphotyrosyl peptide EPQpYEEIPIYL (10 mg ml⁻¹ in 40 mM BES, pH 7.0), which was synthesized and purified as described⁹. Crystals were grown using the hanging drop/vapour diffusion method by combining 2 µl protein/peptide solution with 2 µl of a well solution containing 25–35% PEG 2,000, 4,000 or 8,000, 50 mM MgCl₂ and 100 mM sodium acetate, pH 4.6. Clusters of long, thin rod-shaped crystals of orthorhombic space group *P*2₁2₁2 (a = 61.31, b = 57.31, c = 31.18) grew overnight at 22 °C to maximum dimensions of 0.1 × 0.03 × 1.0 mm. Essentially complete data were collected from two crystals to 2.2 and 1.8 Å resolution using a MAR Research image plate scanner mounted on an Elliot GX-13 rotating anode generator equipped with double focusing mirror optics. Images were processed with the image plate version of the MOSFLM package³². 47,097 fully recorded observations of 9,956 unique reflections were scaled and merged with CCP4 programs³³, yielding a merging *R*-factor of 8.4% on intensities for all data to 1.8 Å. Merged data were 99% complete to 2.2 Å and 85% complete between 2.2 and 1.8 Å. The mean *I*/ σ (*I*) was greater than 2 in the highest resolution shell. Structure determination: Structure factor phases were determined by

molecular replacement³⁴ using the Src SH2/peptide A structure of Waskman *et al.* as a search model¹⁸. Solvent molecules and the phosphopeptide were omitted from the model. Refined atomic temperature factors were retained. Rotation and translation functions and Patterson-correlation (PC) refinement³⁵ were carried out using X-plor³⁶. A cross-rotation function using 8–20 Å vectors and data to 3.5 Å resolution yielded a 9 σ peak. The PC-refined rotation solution was used in a translation search, which yielded an unambiguous 12 σ peak. Inspection of a packing model and of an electron density map, calculated after rigid body refinement, revealed density for the phosphopeptide that had not been included in the model. The model was rebuilt to reflect the amino-acid sequence of the Lck SH2 domain and refined by repeated cycles of manual rebuilding using the program O (ref. 37) and simulated-annealing and positional refinement using X-plor³⁶. The entire phosphopeptide was excluded from the model until after the first cycle of simulated annealing and positional refinement; at this stage the 11-residue peptide was readily built into a 2.2 Å electron density map. Simulated-annealing omit maps³⁶ were used to rebuild several segments of the polypeptide chain, including the N terminus, and the CD and DE turns, where the Lck backbone diverges from Src. The model contains 104 residues (123–226 of intact Lck), the 11 residues of the phosphopeptide and 65 water molecules. The first five residues of the SH2 construct are not visible in the electron density, and E123, the first residue for which main-chain density is visible, is modelled as an alanine. After restrained individual temperature factor refinement, *R*_{cryst} = 22% for 2 σ data to 1.8 Å resolution. The corresponding 'free' *R* value³⁸, calculated using 10% of the diffraction data (selected randomly from eight resolution bins) that had been excluded from all previous refinement calculation, was 31%. The model has reasonable stereochemistry (r.m.s. bonds 0.021 Å, angle 3.7°). It superimposes on the Src SH2 structure¹⁸ with an r.m.s. deviation for C α positions of 1.12 Å.

sequences containing pYMXM or pYVXM (refs 3–7). That is, it appears to have strong specificity for pY+3 and additional selectivity for pY+1. Residue β D5 may be particularly central for peptide recognition (Fig. 1a). The tyrosine in Lck contacts the side chains of pY+1 and pY+3; it constrains the BG loop conformation by accepting a hydrogen bond from the main-chain amide of residue BG5; and it is a ligand of the water that bridges to carbonyl pY+2. A tyrosine is present at β D5 in all members of the Src family, as well as in Abl, Arg, Csk, ZAP70 (C-terminal) and Syk^{1,25–28}. All these proteins have similar sequences in their BG loops (a characteristic Asp-Gly-Leu at BG 2–4) and Ile or Val at β E4 (Fig. 2). We therefore expect that they will have similar pY+3 pockets and a strong preference for hydrophobic side chains of about the same size. Where alignments are clear for other families with more divergent sequences (Fig. 2), the characteristics of the pocket also appear to favour hydrophobic residues at pY+3.

It is clear that a more complete understanding of comparative specificities will require additional structures. An NMR study of the unliganded Lck SH2 domain is in progress (J. Lee, personal communication), and a structure for the Src SH2 domain in complex with the same phosphopeptide used here has been determined at lower resolution²⁹. □

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ERRATUM

The earliest Acheulean from Konso-Gardula

Berhane Asfaw, Yonas Beyene, Gen Suwa, Robert C. Walker, Tim D. White, Giday WoldeGabriel & Tesfaye Yemane

Nature **360**, 732–735 (1992)

THE cat specimen featured on the cover of the issue of 24/31 December 1992, originally referred to *Homotherium* as detailed in the cover caption, was changed to *Dinofelis* sp. aff. *piveteaui* while the paper was in press. The cover shows the cranium listed as *Dinofelis* in Table 2 of this letter.

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Peripherin and the vision thing

A surprising variety of phenotypes are associated with mutations in the retinal protein, peripherin, including the possible human equivalent of the murine rds (retinal degeneration slow) strain.

RETINAL genetics is proving to be exceptionally fertile ground for those probing the mechanisms of visual transduction and retinal degeneration, especially as a growing collection of phenotypes are being associated with specific molecular defects. The classic example is rhodopsin, the archetypal G-protein-coupled photoreceptor with seven transmembrane domains, which is situated in the membranous disks packed together in the rod cells of the retina. More than 30 mutations in the rhodopsin gene are known to give rise to many, but not all, cases of autosomal dominant (ad) and one case of recessive retinitis pigmentosa (RP; reviewed in ref. 1), a group of retinal degenerative disorders characterized by photoreceptor destruction.

Although other X-linked and autosomal RP loci exist, the only other gene which has been formally associated with RP is peripherin/RDS. (This cumbersome title serves to distinguish the retinal peripherin from another human protein with the same name, and also to emphasize its homology with the murine *rds* (retinal degeneration slow) locus). Peripherin/RDS, like rhodopsin, is a transmembrane glycoprotein; but whereas rhodopsin is confined to the rod cells, peripherin/RDS is found in both rods and cones. Two papers in *Nature* in 1991 showed that peripherin mutations are also linked with dominantly inherited RP^{2,3}, but this still leaves most RP cases without a molecular explanation.

Further examination of the peripherin/RDS gene has turned up some rather surprising results, for as explained in three papers in the latest issue of *Nature Genetics*⁴⁻⁶, defects within the peripherin gene account not only for a significant proportion of cases of adRP, but also incidences of macular dystrophy, butterfly-shaped dystrophy of the fovea, and retinitis punctata albescens. Although most of the newly found mutations are missense substitutions (see Table), two are putative null mutations, thereby allowing interesting comparisons to be made with the *rds* mouse.

Nichols *et al.*⁴ examined a three-

generation family in which 11 individuals suffered from butterfly dystrophy, which caused a loss of vision in the centre of the visual field (the fovea), associated with a spattering of large yellowish deposits at the level of the macular retinal pigment epithelium. Scrutiny of various candidate genes quickly identified a mutation within the peripherin/RDS gene (Gly167Asp), which segregated

Newly discovered mutations in peripherin/RDS, and the associated phenotypes

Trp25stop	Retinitis punctata albescens ⁵
ΔCys118	ad retinitis pigmentosa ⁶
Gly167Asp	Butterfly dystrophy ⁴
Arg172Glu	Macular dystrophy ⁶
Arg172Trp	Macular dystrophy ⁶
Tyr258stop	Macular dystrophy ⁶

perfectly with the disease.

Wells *et al.*⁶ examined 58 unrelated individuals with various forms of RP or macular degeneration and identified four distinct mutations among five people affected. One of these was an adRP patient with progressive loss of peripheral vision and, like another patient², has the same single amino-acid deletion (see Table); in contrast, the other three mutations occurred in patients with macular dystrophy who suffer from central vision loss but whose peripheral and night vision is unaffected. One of these mutations was a nonsense mutation in a mildly affected patient with adult vitelliform macular degeneration, which the authors suggest is a putative null allele of the peripherin/RDS gene. Strikingly, this patient also has a yellow deposit in the fovea, apparently at the level of the retinal pigment epithelium.

A key question that arises is which of these human phenotypes directly corresponds to the murine *rds* phenotype, caused by the insertion of a 10 kilobase fragment in the *rds* gene, probably producing a null allele. Although Wells *et al.* speculate that their premature termination mutant at residue 258 may be analogous to the *rds* mouse (because missense mutations or single amino-acid deletions as found in adRP are unlikely to give rise to a null allele), Kajiwarra *et al.*⁵ suggest a different phenotype. They too have detected a premature stop codon in the peripherin/RDS gene caused by deletion of two base pairs

caused by deletion of two base pairs much earlier in the sequence, at codon 25, but in this case the patient suffers from retinitis punctata albescens. This disorder, like RP, is a progressive retinal degeneration, but it differs from RP in that yellow-white subretinal deposits are present. Although the origin of these deposits has yet to be linked conclusively with the peripherin mutation, it is tempting to speculate, in light of the amino-terminal location of the stop codon, that this putative null allele is the human equivalent of the *rds* mouse.

The genes for two human macular diseases (Best's disease^{7,8} and North Carolina macular dystrophy⁹) have recently been mapped. However, the peripherin/RDS studies are the first documented mutations associated with this phenotype, and could prove to be a useful genetic model for a much commoner form of blindness in older people known as age-related macular degeneration. Less clear is why certain mutations give rise to such distinct patterns of retinal degeneration. It is possible that the macular degeneration mutations interfere with the dimerization of peripherin/RDS molecules, or other types of protein-protein interaction. The expression of peripherin/RDS in both rods and cones, in contrast to rhodopsin, is also likely to be significant in explaining the range of phenotypes.

The variety of phenotypes associated with peripherin/RDS abnormalities provides yet another example of the diverse results of mutations within a single gene. Other examples include the collagen II gene, which can give rise to chondrodysplasia¹⁰ and Stickler syndrome¹¹, and the paired box gene, *PAX3*, defects in which cause both Waardenburg syndrome and the paediatric solid tumour, alveolar rhabdomyosarcoma¹².

Kevin Davies

Kevin Davies is Editor of *Nature Genetics*.

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Also in this month's *Nature Genetics*: adenoviruses deliver genes to the brain; an improved mouse model for Lesch-Nyhan disease; mapping the gene for a hereditary form of stroke; and community screening for cystic fibrosis.